Cell lineage tracing during Xenopus laevis tail regeneration

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Acknowledgment

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Abstract

The tail of the *Xenopus* tadpole will regenerate following amputation, and all three of the main axial structures: the spinal cord, the notochord, and the segmented myotomes, are found in the regenerated tail. We have investigated the cellular origin of each of these three tissue types during regeneration.

We produced *Xenopus laevis* embryos transgenic for CMV (Simian Cytomegalovirus) promoter driving GFP (Green Fluorescent Protein) ubiquitously throughout the embryo. Single tissues were then specifically labelled by making grafts at the neurula stage from transgenic donors to unlabelled hosts. When the hosts have developed to tadpoles, they carry a region of the appropriate tissue labelled with GFP. These tails were amputated through the labelled region and the distribution of labelled cells in the regenerate was followed. We have also labelled myofibres using the Cre-lox method.

The results show that the spinal cord and the notochord regenerate from the same tissue type in the stump, with no labelling of other tissues. In the case of the muscle, we show that the myofibres of the regenerate arise from satellite cells and not from the pre-existing myofibres. This shows that metaplasia between differentiated cell types does not occur, and that the process of *Xenopus* tail regeneration is more akin to tissue renewal in mammals than to urodele tail regeneration.

I-Introduction

Tail structure and function

A post-anal tail is a feature of vertebrates and lower chordates in contrast with the remaining Bilateria. The tail is a major region of the body of most vertebrates, constituting that part of the body posterior of the caudal opening of the digestive tract. In fish and water-dwelling amphibians it is chiefly used for locomotion (Fig.I1).

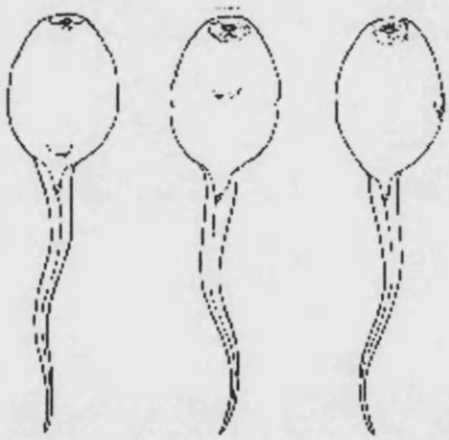
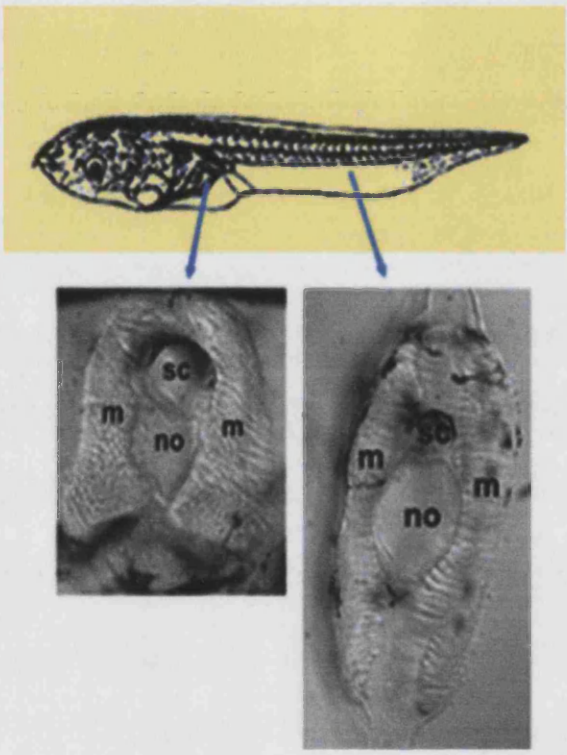


Fig.I1 Anuran *Bufo bufo* swimming tadpoles.

The tail of all vertebrates consists almost exclusively of a caudal continuation of the body wall: of muscle for power, of nervous system for innervation, of vertebral column for muscle support, of dorsal aorta for arterial blood and of caudal vein for vascular drainage. Two of the three orders of modern amphibians are classified on the basis of presence or absence of the tail: the Urodela or Caudata (tailed amphibians); and the Anura (frogs and toads) which exhibit a locomotor tail prior to metamorphosis, when as water-dwelling tadpoles, they need it most. At vertebrate embryonic stages a notochord is found rather than vertebral column. In larvae of the anuran amphibians, the tail is a transitional structure which becomes re-absorbed during metamorphosis, mediated by thyroid hormone action. Nevertheless the tail of *Xenopus laevis* (anuran amphibia) tadpoles retains the core organization common to the other vertebrates: a spinal cord and notochord flanked by paired myotomes in continuation with the axial trunk (Fig.I2).

The tails of reptiles, birds and mammals are phylogenetically ancient structure originally adapted for swimming, but now utilized for distinct purposes. Modern birds have reduced the tail region to a nubbin but the earliest known birds had long tails. Mammals exhibit elongated, prehensile tails (monkeys), foreshortened tails (hamster), flyswatters (cattle), balancers (squirrels), and organs of defence (porcupines) (G.C. Kent, 1965). Even humans exhibit a tail in early embryonic life, vestiges of which may be observed on any human skeleton as the lowest three or four caudal (coccygeal) vertebrae.

Fig.I2 *Xenopus laevis* tail section at different levels: (m) muscle, (no) notochord and (sc) spinal cord.



Birth defects can occur as a consequence of developmental anomalies during secondary neurulation (see bellow): like spina bifida or other neural tube defect (Fig.I3) (Copp and Brook, 1989; Copp et al., 1988; Dryden, 1980).

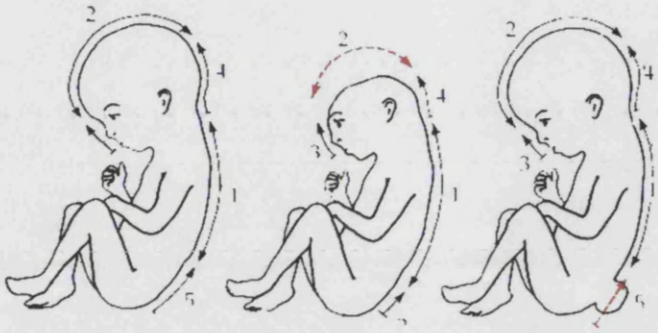


Fig.I3 Sequence of the neural tube region closure: **(A)** Normal; **(B)** Anencephaly, due to lack of neural plate fusion in the region 2; **(C)** Spina Bifida, due to the lack of fusion in the region 5 (or to the lack of closure of the posterior neuropore) (Van Allen et *al.*, 1993).

Vertebrate tail development

The embryogenesis of the tail has been studied for many centuries starting with Von Kolliker (1879, 1884, 1889) followed by Holmdahl (1925,a,b,c) and Vogt (1926). Holmdahl theorized that the development of the vertebrate proceeds in two phases: primary body development and secondary body development. During primary development the anterior posterior axis, with the structure of head and trunk, differentiate from the three embryonic germ layers established by the gastrulation movements. During secondary development, for Holmdhal occurring in the caudal part of the embryo, the embryonic germ layers are not involved in the tail formation. Instead, a protruding pluripotent mass of mesenchymal cells (blastema) localised in the posterior end of the embryo, the so called tail bud, gives rise directly to all the tail structures. Those blastema-like

cells have been theorized behave like the limb bud during limb development. Von Kolliker, in agreement with Holmdahl 50 years earlier, recognized that the spinal cord of the tail tip became distinguished from mesoderm in a process called secondary neurulation. Thus secondary neurulation has been described in many vertebrate groups: lampreys, neopterygian fishes (*Teleostei, Lepidosteus, Amia*), sarcopterygian fishes (*Lepidosiren, Protopterus*), frogs, chick and mammals (Kingsbury, 1932; Criley, 1969; Hughes and Freeman, 1974; Shoenwolf and De Longo, 1980; Balinsky, 1981; Nakao and Ishizawa, 1984; Shoenwolf, 1984; Griffith, 1992; Papan and Campos-Ortega, 1994; Shmitz et al., 1994, Hall, 1998; Beck and Slack, 1998). In secondary neurulation, cells from the tail bud condense to form a solid cord tissue called the medullary cord or neural keel. Following this, tissue begins to cavitate with the formation of a hollow and eventually forms the neural tube (Fig.I4). This process is utilized by some vertebrates classes as the neurulation mechanism for the entire length of the body: e.g. neopterygian fishes and lampreys (Nakao and Ishizawa, 1984; Reichenbach et al. 1990; Schmitz et al. 1993; Papan and Campos-Ortega, 1994). A number of small cavities in the central canal, or secondary neurocoel, are involved in the cavitation of the neural keel forming the spinal cord along the entire body length (Hughes and Freeman, 1974; Schoenwolf and De Longo, 1980). However in regard to frogs, birds and mammals the secondary neurulation takes place only at the caudal end of the embryo, after primary neurulation ending at the level of the trunk (Nievelstein et al., 1993).

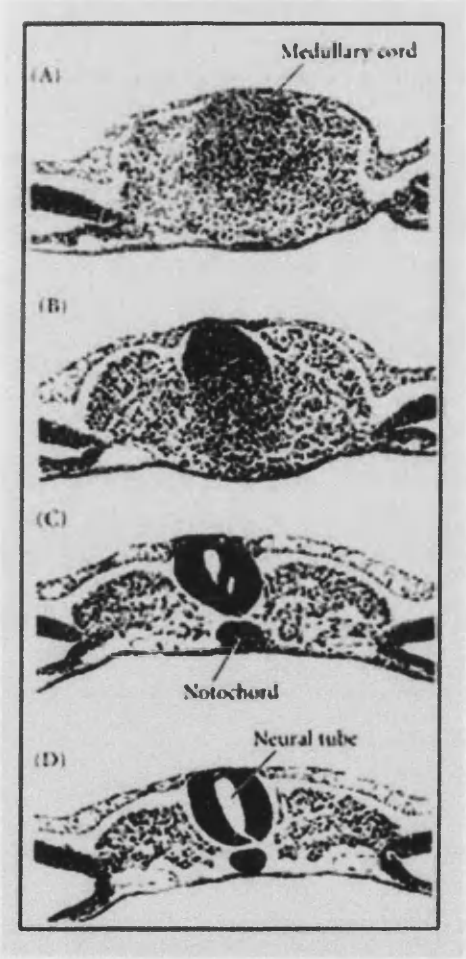
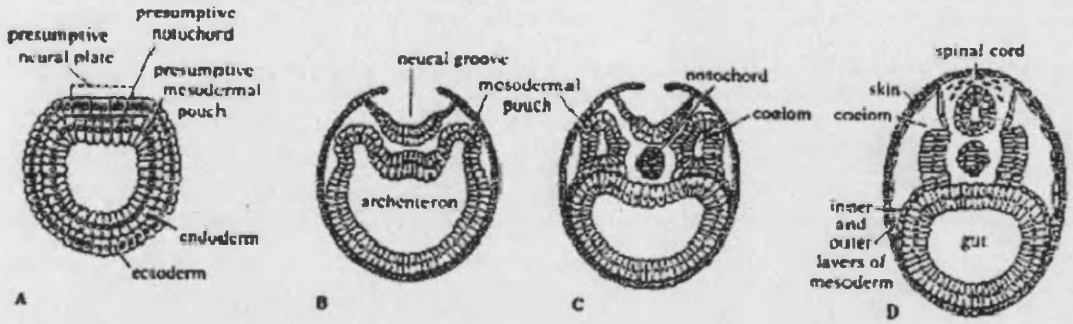


Fig.I4 Caudal view of the second neurulation: **(A-B)** Formation of the solid medullary cord in the caudal portion of the embryo; **(C)** Cavitations of the medullary cord with the formation of the neural tube **(D)** (Gilbert, 2000).

During gastrulation the embryo undergoes a precise sequence of tissue reorganizations that will give rise to the blastopore, an important organizer for body formation. After closure of the blastopore, the neural epithelium derived from the ectoderm becomes thicker forming the neural plate, resulting from a complex series of induction between ectoderm and mesoderm. This plate rapidly bends into a groove and then folds in the neural tube by fusion of its dorsal borders (Fig.I5).



Neurulation

Fig.I5 Schematic view of the primary neurulation in lower vertebrates: **(A)** Formation of the neural plate; **(B)** Folding of the neural plate to form the neural groove and neural folds; **(C)** Closure of the neural groove to give rise to the formed neural tube; **(D)** Ending of the process with the complete mature neural tube formed with the underlying notochord and flanking somites.

This process has been called primary neurulation (Schoenwolf and De Longo, 1980; Shoenwolf, 1984 and Shoenwolf and Smith, 1990). In amphibians and mammals this phase ends when the posterior neuropore closes. The neural tube closes over the blastopore to create the tail bud consisting of three regions: the caudal notochord, the caudal neural tube and, above these, a region of mesoderm derived from the caudal part of the neural tube (Shoenwolf and De Longo, 1980, Schmitz et al., 1993; Beck and Slack, 1998). After posterior neuropore closure, the tail bud drives the morphogenesis and growth of the caudal half of the body, the process designated as secondary body development (Holmdahl, 1925a). When cavitation of the secondary neural tube is achieved, the primary and secondary neural tube are morphologically similar and the

boundary between the two types of neurulation vanishes. The "tail bud" generates not only the tail, but all the tissues located in the caudal half of the embryo (Holmdhal, 1925a,b,c; Criley, 1969: Shoenwolf, 1977;1978;1979; Costanzo et al.1982) hence the name "bourgeon tronco caudal" given by Pasteels (1939) to this structure. But in *Xenopus laevis* development, and probably other anurans as well, the tail does not come just from the tail bud but from the displacement movement of the trunk (Tucker and Slack, 1995a).

Vogt and Pasteels did not share the same interpretation proposed by Holmdahl regarding the composition of the tail bud. Walther Vogt (1926) proposed a new model in regard to tail development highlighting the "unity and continuity" of the vertebrate body plan, and considering that the tail arises from continuation of the morphogenetic processes of the gastrulation. Later Pasteels (1942, 1943) elaborated further Vogt's model, in particular identifying in the tail bud a structure that he called "charnière chordo-neurale" or chordo neural hinge (CNH). This structure contributes to the notochord and spinal cord of the tail and it is the direct descendant of the dorsal lip of the blastopore. He considered the tail bud not as the homogeneous blastema proposed by Holmdahl, but as the result of a mosaic of distinct cell population having their origin from the gastrulation movements. More recently, fate mapping, tissue extirpation and tissue transplantation techniques were used to show that different regions of the tail bud are not only fated, but also committed to produce different tissue types well before tail outgrowth (Gont et al 1993; Tucker and Slack, 1995a).

Furthermore Pasteels (1942) showed that the cell proliferation rate in the tail bud was not significantly higher than other embryo body regions, as predicted by the blastema model. Davis and Kirshner (2000), in *Xenopus* embryo, followed the fate of a small neighbourhoods of developing photo-activated fluorescent cells in the tail bud, trying to support the Holmdahl point of view of the multipotent nature of the tail bud cells. Nevertheless since they always labelled clusters of cells there is not prove to conclude this. In relation to higher vertebrates, Griffith et al. (1992) reviewed the development of vertebrate tail bud relying mostly on the work of Shoenwolf and concluded that it is indeed a homogenous mass of blastema-like cells, as originally described by Holmdahl. However, zebrafish and chick tail buds do not show elevated cell proliferation rates (Shoenwolf, 1977; Mills and Bellairs, 1989; Kanki and Ho, 1997). and the interpretation of Vogt and Pasteels found new life in the work of Catala et al. (1995). They performed the same studies as Shoenwolf in the early chick embryo grafting selective regions of the tail bud rather than the entire bud. With this high resolution technique the early chick tail bud was shown to contain several mesenchymal cell populations arising from gastrulation movements. They described the equivalent structure to the CNH of the *Xenopus*, which is derived from the Hensen's node, and gives rise to the notochord and spinal cord. Other findings support Catala's work, showing the heterogeneity of the tail bud in terms of gene expression domains (e.g. *Gnot1, Tbx6L*) running from the primitive streak into distinct regions of the tail bud (Knezevic et al., 1998). Moreover they have also demonstrated organizer

activity and ingressive gastrulation-like movements occurring in the tail bud. The studies on chick embryos shed light also on the cellular origin of the incipient neural tube during secondary neurulation. Catala et al. (1996) and Le Douarin et al. (1998), performing experiments using quail-chick chimeras, demonstrated that epithelial cells from neural plate give rise to the medullary cord. Therefore it is reasonable to regard caudal development in avian embryos as continuous with that occurring more anteriorly in the embryo. The zebrafish tail bud, like that of birds, consists of a heterogeneous cell population and gastrulation-like cell movements still occur at the caudal terminus of the embryo (Kanki and Ho, 1997). Nevertheless some gene expression involves genes expressed almost exclusively in the tail bud: e.g. *snail* (Thisse et al., 1993), *eve1* (Joly et al., 1993) and *caudal* (Joly et al., 1992),. Therefore the zebrafish tail bud shows some developmental features consistent with both Holmdahl's and Vogt's interpretation. The mammalian tail bud is histologically a homogeneous mass of mesenchymal cells overlain by a ventral ectodermal ridge (VER). The VER has been compared with the apical ectodermal ridge (AER) of embryonic limb bud for its histological similarity (Gruneberg, 1956) and for its gene expression repetoire: specifically *Msx1*, *Wnt5a*, *BMP2* and *FGF17*. Nevertheless the only inductive function seems to be regarding the *BMP* antagonist *noggin* expression in the ventral tail mesoderm (Goldman et al., 2000). Functions for the other genes have yet to be determined, but their localized expression suggest that VER , like the limb AER, might be a source of possible molecular signals that regulate tail

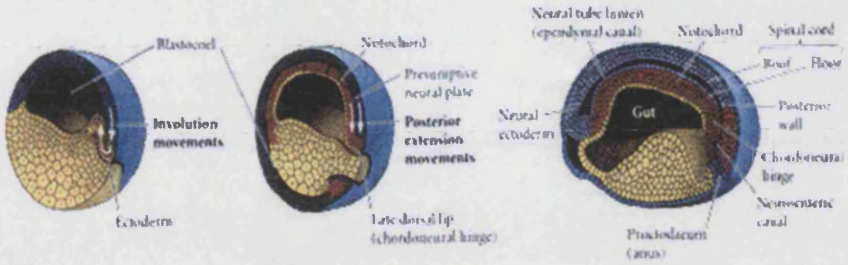
bud mesenchyme. Several fate mapping experiments showed that the tail bud results from materials coming from the primitive streak and Hensen's node, indicating that anterior developmental movement (gastrulation-like) may continue into the caudal portion of the embryo (Tam and Tan, 1992; Wilson and Beddington, 1996). Furthermore several marker genes (*Wnt5a, Tbx6, Hoxb1*) have been followed during gastrulation in the primitive streak and Hensen's node and followed into distinct tail bud regions (Gofflot et al., 1997). The presence of a tail bud with similar gene expression domains among all vertebrates suggests that the mechanism of tail formation is well conserved, although the contribution of the tail bud to axial structures may vary in terms of the level at which it commences. The recent interpretation of Gregory Handrigan (2003) supports this idea elaborating, on the basis of studies in a cephalocordate (Amphioxus) and a urochordate (*Tunicates*), the theory of a common ancestor without a tail bud presenting exclusively primary neurulation for neural tube formation. In fact Shubert et al. (2001) showed that amphioxus (the closest living organism to the ancestral vertebrate) has a CNH, like zebrafish, frogs and mammals, but how this structure contributes for tail elongation is not yet understood. Furthermore Satoh (1994) and Katsuyama et al. (1999) demonstrated that the urochordates (sea squirts) do not have a real tail bud, but rely exclusively on the movement of pre-existing cell populations for tail elongation. Thus Handrigan describes the developmental ambiguity of the two hypothesis regarding tail development as "discordant harmony", because the tail bud can not be the rigid mosaic proposed

by Vogt neither the homogeneous blastema-like tissue proposed by Holmdahl. As this classical distinction depends only on the interpretation of descriptive data, further progress in understanding the mechanism of tail development will requires the use of cellular and molecular techniques.

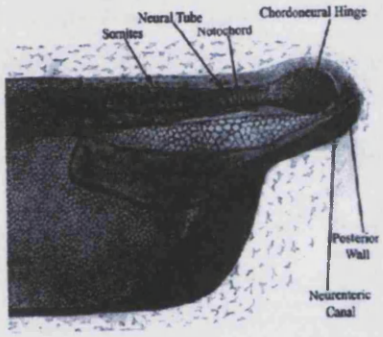
Xenopus laevis tail development

The *Xenopus* tail is defined as the tissue located posterior to the proctodeum. The anterior movement of the proctodeum during body extension results in a displacement of the embryonically derived trunk tissue into the proximal two thirds of the tail axial tissue. In the caudal end of the embryo the so called tail bud gives rise to about the distal third of the tail. Within the prospective tail bud region the neural plate is not entirely neural, because its most posterior part is destined to form tail somites (Bijtel, 1931; Smithberg, 1954; Woodland and Jones, 1988; Tucker and Slack, 1995a). The caudal part of the axial system which will take part in the formation of the tail bud develops relatively late, although it becomes distinct at stage 24 NF (Niewkoop and Faber) and starts elongation at stage 28 NF. The tail bud is composed, using the anatomical nomenclature of Gont (1993), of: the neural tube, notochord, chordoneural hinge, neuroenteric canal, mesoderm and posterior wall (Fig.I6).

Fig.I6 Amphibian neurulation and tail development: **(a)** Early gastrula **(b)** Late gastrula **(c)** Early tail bud (Scott F. Gilbert, Developmental biology) **(d)** Late tail bud (Kirshner and Davis, 2000).



(a) (b) (c)



(d)

The neural tube is formed by the closure of the neural plate in the caudal region; the chordoneural hinge by the dorsal lip of the blastopore, remnants of the Spemann's organizer. It is continuous both with the floor plate of the neural tube and the underlying notochord. The posterior wall is derived from the lateral blastoporal lip, this contains most of the mesodermal component of the posterior neural plate and it has lateral continuity with the chordoneural hinge. The neurenteric canal is an intriguing structure that did not receive much attention from previous embryologists. The formation of the neurenteric canal in *Xenopus* can be followed by simply observing dechorionated neurulae. During the neural

plate stage, the blastopore becomes elongated forming a narrow slit. Cells of the collar of the surrounding blastoporal region (*Xbra* positive) rise up on either side of the central portion of the slit and eventually fuse in the dorsal midline. As a result, a canal is formed that is opened anteriorly at the neural plate and posteriorly at the future anus. In a second set of movements, the neural folds rise and form the neural tube, enclosing the anterior opening of the neurenteric canal, but not the posterior opening. In this way, the lumen of the spinal cord (the ependymal canal) becomes connected to the anus via the neurenteric canal. Thus, the posterior wall of the neurenteric canal is formed from *Xbra* positive cells of the lateral portion of the circumblastoporal collar. While this description does not differ significantly from those provided for *Rana* by previous workers (Kerr, 1919; Pasteels, 1943), there is not evidence in the literature for the existence of a neurenteric canal in chick and mouse embryos despite its central role in the development of the tail. The neurenteric canal probably represents an ancestral chordate feature, since it is present in amphioxus (Hopper and Hart, 1985), shark (Kingsbury, 1932), gecko (Kerr, 1919), turtle (Yntema, 1968) and human embryos (Moore, 1977). Then Gont (1993), also reviewed by De Robertis et al. (1994), elegantly investigated the structure of the tail bud and the movements leading to the tail formation and elongation, by associating cell tracers and gastrulation molecular markers as the homeobox factor *Xnot2* (blastopore dorsal lip) and the T-box factor *Xbra* (entire blastopore). The homeobox factor are a family of transcription factors, containing a homeodomain

as DNA binding sites. This is an approximately 60 amino acid sequence containing many basic residues forming a helix-turn-helix structure to bind specific DNA sequences. The homeobox was given its name because it was initially discovered in homeotic genes. The T-box factors are a family of genes sharing the DNA binding domain similar to the prototype gene product knows as "T" in the mouse and *Brachyury* in other animals. Gont et al followed the expression of those two genes during gastrulation into two distinct populations of the *Xenopus* tail bud: the CNH and the posterior wall. These two population are separated by the neurenteric canal and contribute to the differentiation of distinct elements within the tail: notochord and ventral spinal cord from the CNH, the somites from the posterior wall. They also demonstrated that the tail bud takes its form directly from the lips of the blastopore, and that it retains organizer activity. By implanting the CNH region from early tail bud (st. 25 NF) and later tail bud (st. 35 NF) into the blastocoel of early gastrula embryos they showed tail like structure developing in the host . In contrast, implants of prechordal plate from the same stage embryos produced structures with anterior characteristics, such as cement glands. The structures resulting from both stages of CNH implants are typical tails, as indicated by the presence of dorsal and ventral fins and of well organized notochords flanked by paired myotomes and neural tissue. A fundamental property of the organizer is the recruitment of cells from the host into a twinned axis (Spemann and Magold, 1924), and Gont et al showed effectively the recruitment of host tissues to form axial structures by the

graft of CNH. This was done by labelling the grafts with fluorescein dextran amine (FDA) or with DiI and showing that labelled cells contributed to only a small part of the secondary tail. The main mechanism driving the involution of the mesoderm through the blastopore lip is convergence and extension (Keller and Danilchik, 1988; Keller et al., 1992; Shih and Keller, 1992). The cells converge on the dorsal midline (both in the mesodermal and the ectodermal layer) they intercalate with each other, resulting in an elongation that is the main engine of gastrulation movements. To investigate whether such movements continue during tail formation, Gont et al. labelled late blastoporal lips at dorsal and lateral positions and allowed the embryos to develop to tailed swimming tadpoles. This showed that labelled cells were interspersed throughout the notochord, extending to the tip of the tail. In the posterior notochord, labelled cells were interspersed with unlabelled cells derived from other caudal region. This pattern of interspersed cells is diagnostic of cell intercalation in *Xenopus* (Niehrs and De Robertis, 1991). Furthermore a row of muscle cells were labelled in the somites: the cells become increasing longer in the older, more anterior somites. This myotomes elongation could provide a second mechanism which may contribute to tail elongation in frogs, as has been proposed by Elsdale and Davidson (1983). Thus the caudal part of the *Xenopus* embryo body develops in a continuous manner with the anterior part of the embryo formed during gastrulation, and intercalation movements continue also at late stages of tail development, even if the contribution to the tail elongation is not yet known.

Tucker and Slack (1995a,b) further supported the above idea by fate and specification maps showing the contribution of trunk cells, located anterior to the proctodeum, to the formation of the tail. The conversion of the trunk cells into tail occurs through the displacement of the anus to a more rostral position during tail development. Therefore those trunk cells constitute part of the "tail-forming region" of the neurula. They also demonstrated that the axial structures of the tail: neural tube, notochord and somites, derive from distinct mosaic of cell populations established during gastrulation. Furthermore they showed that is the formation of the tail bud needed the interactions of three specific regions around the blastopore lips called N, M & C. They proposed this model based on the behaviour of embryos undergoing exogastrulation. Amphibian blastulae when placed in an isotonic salt solution undergo an incorrect gastrulation movement resulting in the eversion of the meso-endoderm instead its invagination into the interior. The gene expression patterns show that mesoderm and endoderm are well patterned and comprise all tissue structures of the normal embryos except for certain anterior neural elements (Ruiz i Altaba A., 1994). However the exogastrula does not produce any tail, because the NMC interaction in the caudal region cannot take place. The three regions are: the most posterior neural-plate fated to form tail somites (M), the neural plate immediately anterior to M (N) and the underlying caudal notochord (C). To initiate tail bud formation, according to the model, C must underlie the junction of N and M which subsequently forms

the tip of the tail, this interaction between NMC regions leads the tail bud specification occurring at about the end of gastrulation (Fig.I7).

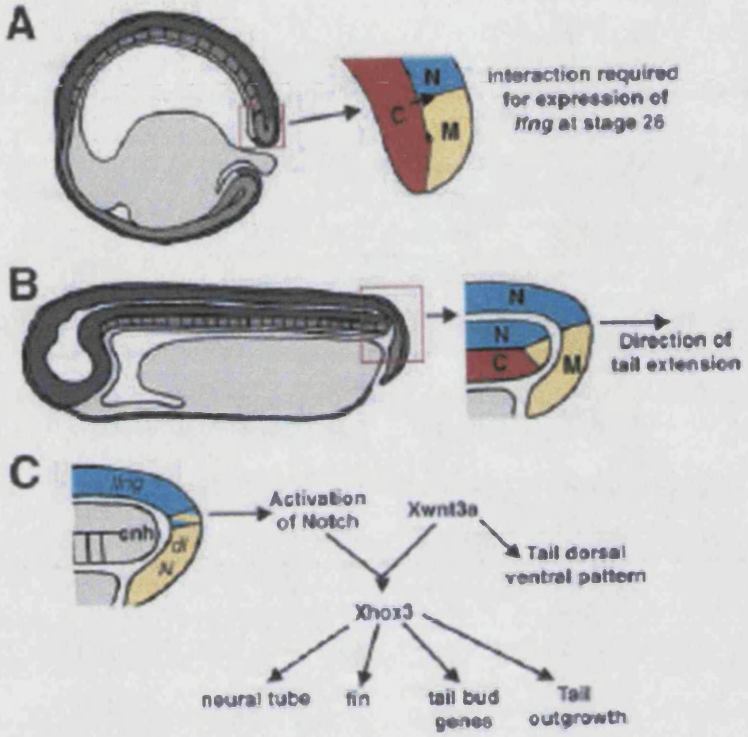


Fig.I7 Schematic representation of the development of the Xenopus *laevis* tail bud in longitudinal section: **(A)** Tail bud formation, stage 13 NF, at the end of gastrulation **(B)** Tail bud, stage 26 NF, at the closure of the blastopore with the formation of the neuroenteric canal **(C)** Enlarged posterior region at later stage where are shown the contacts between N (Neural Plate), M (Neural Playe deived Mesoderm) and C (Caudal chordamesoderm) regions to initiate the tail bud formation and the specification of specific structure resulting in a particular gene expression (Beck and Slack, 1999).

Although there are not specific markers for these three regions, Beck and Slack (1998) later showed that *Xlim1*, is expressed in the posterior part of the notochord and dorsal lip of the blastopore at the end of gastrulation and in the

chordoneural hinge and in the posterior tip of the differentiated notochord at later stages. *Xlim1* belongs to the LIM family of homeodomain factors: characterized by a cysteine-rich zinc-binding region responsible for protein-protein interaction and DNA-binding homeodomain together (Taira et al., 1992). *Xcad3*, a transcription factor homologue of *Drosophila caudal,* is exclusively expressed in the posterior of the *Xenopus* embryo: during gastrula stage in the posterior neural plate and persisting, later, in the tail bud at level of the posterior wall and posterior neural tube (Northop and Kimelman, 1994; Pownall et al., 1996). In addition to the results already showed by Gont and colleagues (1993), *Xnot2* and *Xbra* present expression in other distinct region of the tail bud, respectively: dorsal blastporal lip (gastrula stage) and CNH (tail bud stage); all blastopore (gastrula stage) and all tail bud (tail bud stage). Therefore the above described molecular markers, labelling different distinct cell populations, do collectively enable the definition of N, M and C regions. The posterior part of the notochord differs from the remaining, corresponding to the C region, by the restricted expression of *Xcad3* and *Xlim1*, also the posterior ventral neural tube (N) expresses *Xnot2* but not *Xlim1* or *Xbra*. Although there is no specific molecular marker for the M region at stage 13 NF, the absence of expression of *Xlim1* at the tip of chordoneural hinge during outgrowth indicates the presence of residual M domain in the hinge (Beck and Slack, 1998). A previous study, earlier described, provided evidence for three separate domains established at the time of tail bud formation and maintained in the derivatives of these domains

during tail outgrowth on the basis of *Xnot* and *Xbra* expression (Gont et al., 1993). These are the CNH expressing *Xbra* and *Xnot2*, the posterior wall of the neurenteric canal that express *Xbra* but not *Xnot2*, and the ventral neural tube which express *Xnot2* but not *Xbra*. Beck and Slack (1998), moreover demonstrated the presence of further domains during early extension of the tail by *in situ* studies of a wide range of genes. The expression patterns enabled seven domains to be identified. As with all *in situ* hybridisation studies, these regions are defined on the basis of gene expression, and this does not assure the presence of an active protein. The first new domain extends from the posterior dorsal neural tube to the tip of the extending tail, the posterior wall of the tail bud. This region, called dorsal roof of the tail bud (DR), is marked by the expression of transcripts of three secreted proteins: *lunatic fringe*, *Xwnt3a* and *Xwnt5a*. *Lunatic fringe* is one of a group of genes homologous to *Drosophila fringe* and codes for a glycosyl transferase. It can modify the components of the *Delta-Notch* signalling system such that *Notch* activation occurs only along a boundary between *fringe*-expressing and non-expressing region. *Xwnt3a*, *Xwnt5a* and *Xwnt5b* are the only *wnt* genes expressed in the mouse tail bud (Takada et. al, 1994), suggesting a probable conserved function in vertebrate tail development. The second novel domain is the part of the posterior wall that forms the distal tip of the tail as determined by its direction of outgrowth (T). This domain is marked by a single gene, *Xhox3*, which is a homologue of *Drosophila even-skipped*. The third domain marks the outgrowing posterior tip of

the chordoneural hinge, corresponding to the late axial component of the M region (MH), which expresses *Xbra* but is distinguished from the CNH by a lack of *Xlim1* expression. The final new domain is the posterior notochord (PNC), marked by the expression of the *Xlim1* but not *Xnot2*. Beck and Slack moreover showed the expression of other several genes expressed in the cells of the fin: *Xwnt3a*, *Xwnt5a*, *BMP-2* and *BMP-4*. Although they are not classified as domain of the tail bud, nevertheless the fin domain is adjacent to the DR, T and the posterior wall (PW) domain of the tail bud. Fin genes may therefore play a specific role during tail elongation. Moreover Gawantka et al. (1998) identified genes isolated in a large screening expressing in distinct domains of the tail in order to define nine tail territories. The most of those genes, seven, were already described as mentioned above (Gont et al., 1993; Bech and Slack, 1998); two others are not yet described. The most remarkable regionalization has been displayed for the CNH, which is divided in three anterior-posterior domains; and for the posterior wall divided into three dorsal-ventral and anterior-posterior domains. Gawantka et al. (1998) results confirmed the seven domains described by Beck and Slack (1998), in addition they defined two additional domains in the developing tail bud proper, namely the ventral posterior wall positive for *2.15/Xvent1* and the mid-chordoneural hinge positive for *11D1*.

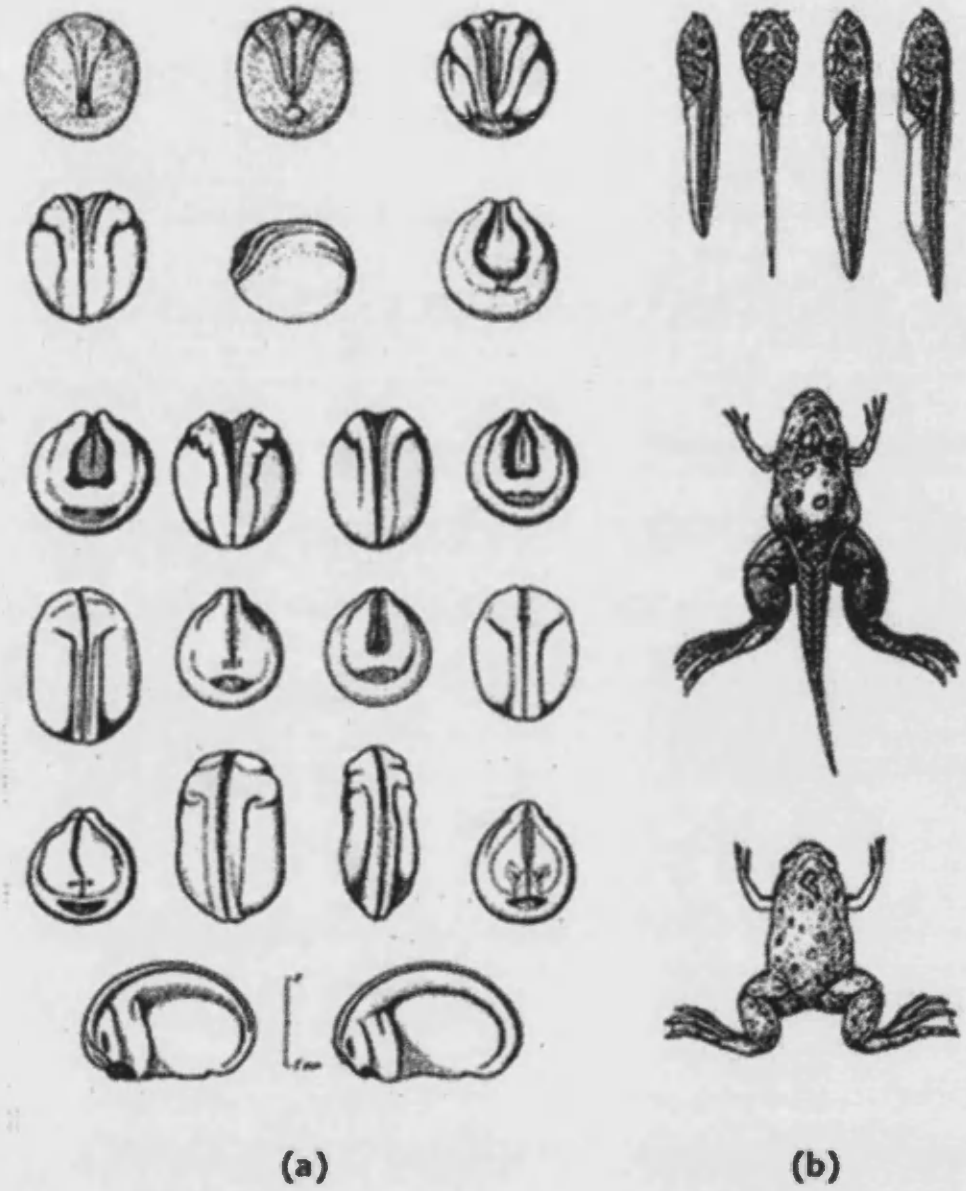


Fig.I8 Niewkoop and Faber (1967) table of Xenopus *laevis* stages of development: **(a)** Neurulation **(b)** Metamorphosis

Metamorphosis

The tail is fated to be reabsorbed during metamorphosis under the action of thyroid hormones (TH) (Fig.I8). The post embryonic period before metamorphosis has been split up by Etkin (1964, 1968) into three phases: pre-metamorphosis (st. 52/55 NF), pro-metamorphosis (st. 55/58 NF) and metamorphic climax (st. 59/66 NF). These three phases are correlated with endocrine changes: the hormone prolactin, which has an anti-thyroid action in larvae, is produced at early pre-metamorphosis, decreasing as the level of thyroid stimulating hormone (TSH) rises during pro-metamorphosis. This increases thyroid activity with a substantial storage of TH within enlarging thyroid follicles. Just before the onset of pro-metamorphosis the thyroid releasing factor (TRF) mechanism becomes sensitive to the initial level of circulating TH, thus the progressive increase in the production of TRF stimulates TSH, which stimulates TH secretion. This leads to an autoactivating effect and further raises the levels of production and secretion of TRF, TSH and TH, with concomitant characteristic and sequential larval morphological changes, such as tail re-absorption during the climax.

General features of regeneration

Aristotle in book II of "*History of animals*" describes for the first time the regeneration process: "The tail of lizards and of serpents, if they be cut off, will grow again". Thousands of years after Aristotle's biological treatises other

scientists were attracted by this fascinating phenomenon in different structures and species: Perrault (1688) lizard tail regeneration, Rèamur (1712) crayfish claw regeneration and Spallanzani (1768) salamander and tadpole limb and tail regeneration (Fig.I9A). This interest was associated with the scientific and social controversy regarding Preformation and Epigenesis. Preformation has been the most widely accepted explanation of the generation of complex animal forms since the late seventeenth century. An extreme representation was the "emboîtment" model, in which successive generation of organisms were encapsulated one within the other. The future generation was seen as a miniaturized organism already preformed at the moment of the creation This accorded with the Cartesian mechanical view of nature and at the same time left God with an active role. Given the theological and philosophical notions of predestination of the Calvinist doctrine, both Jansenist and Protestant accepted the preformationist theory of generation (Roger, 1963; 1980). The Epigenetic theory is typically in opposition to pre-formation, giving greater play to nature in the generation of complex bodies by the action of self-regulating dynamics or of an autonomous but indeterminate "force". Important in the birth of this theory was the influence of chemistry and physics which relied on "attraction forces" such as gravitation. De Maupertuis (1752) regarded the pre-formation model as untenable: "Why shouldn't a cohesive force, if it exists in Nature, have a role in the formation of animal bodies?". Meantime Trembley (1744) demonstrated how a bisected hydra gives rise to the production of two complete animals, thus

animal regeneration became a central issue in the confrontation between pre-formation and epigenesis. The regeneration of appendages in urodeles revealed that a quadruped could regenerate and restore missing parts completely with form and structure. Moreover, regeneration of a quadruped appendage was problematic because in seemed in conflict with the prevailing concept regarding the generation of higher organisms. The epigenetic interpretation of regeneration also represented a challenge to certain aspects of social and political life consisting in the idea of the natural order. Regeneration came to be an essential part of the epigenesis/pre-formation debate, therefore constituting a formative element of eighteenth century thought in a period of social and intellectual revolution (Dinsmore, 1996). 500 years earlier than Aristotle's biological treatises (*Parts of Animals*, *History of Animals* and *Generation of Animals*) the regeneration phenomenon had already attracted pre-scientific interest, or at least curiosity. Hesiod's Theogony (eight century BCE) contains the fantastic imaginary story of the Hydra's capacity to regenerate multiple replacement of lost heads (Fig.I9B). As seen above, in the eighteenth century, Trembley found a small coelenterate, called "polypes" first and later "Hydra", that actually had that incredible capacity to regenerate two complete animals from a bisected one. Hesiod also write about the legend of Prometheus' liver regeneration; after punishment from the gods for fire stealing, his liver was plucked out each day by an eagle, to regenerate during the night (Fig.I9C). The fact that the liver does show this incredible capacity, even in humans (although not at that rate),

represents a coincidence, a convergence of myth and reality. There are not reasons to believe that these histories do not represent just fascinating mythology transcribed by oral tradition. The interest in regenerating capacity must have occupied the imagination since someone survived the first accidental amputation and it is still being investigated because of its potential use in modern regenerative medicine.

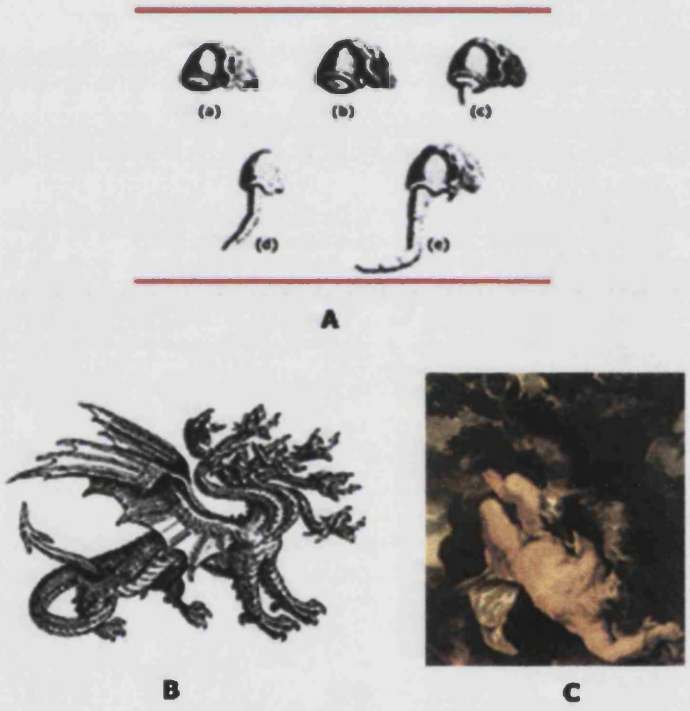


Fig.I9 **(A)** Rèamur (1712) diagram of crayfish claw regeneration; **(B)** Representation of the Hydra, animal with multiple heads; **(C)** Painting representing Prometheus liver plucked out by the eagle.

Regeneration in its broadest sense comprises all the intracellular turnover of bio-molecules and organelles in order to answer demands of homeostasis. To survive, all organisms in some way or another express this constitutive type of

regeneration to maintain the integrity of protein synthetic machinery and to support the recycling of plasma membrane. In fact loss or impairment of those capacities leads to death. This represents regeneration at a molecular level, however the process of biological regeneration in the more common sense of the term relates to the mechanism of repairing or replacing damaged or lost parts. The structure undergoing regeneration (molecules, cell, tissue and organs) may do so as a matter of normal, physiological turnover of specific components, or alternatively following an injury. Thus physiological regeneration occurs during normal life of the organism to repair structures exposed to sustaining wear or abrasion. Examples include the continuous proliferation of the cells of the basal layer of the skin epidermis in order to replace and regenerate the superficial outer layer, and the replacement of the cell population lining the gut by renewal from crypt proliferating stem cells. Other structures also undergo periodic shedding and replacement: e.g. hair and feathers in vertebrates, or skin scales in reptiles. Invertebrates also show physiological regeneration: as in the hormone-driven growth of arthropods accompanied by ecdysis. Nevertheless the regeneration in the sense of a complete structure and functional replacement of a missing part varies remarkably among phyla. The regenerating processes are divided in two major types: morphallaxis and epimorphosis (Morgan, 1901). Morphallaxis is a feature of more primitive organisms; they can restore lost parts by reorganizing the remaining structures into a complete functional new individual. Hydra and some planarian flatworms exemplify this process, replacing

damaged or missing parts respectively by cell movements and the proliferation of parenchymal cells, the so called neoblasts. Those are an interstitial population of reserve cells that reconstruct the lost parts after wound closure by the wound healing epithelium. Wound healing is almost universal, but injury-induced reparative epimorphic regeneration occurs only sporadically among and within different groups of animals and it might follow different pathways for the purpose of restoring lost structure and function of the organism. Epimorphic regeneration represents the process by which the lost structures are reconstituted by the dedifferentiation and proliferation of the remaining cells. in the process called dedifferentiation many tissues surrounding the damaged surface lose cytoplasmic specialization and regain mitotic activity. They form a mass of undifferentiated pluripotent and actively proliferating cells, the blastema. This phase of regeneration requires close epithelial-mesenchymal communication; in fact wound healing has an important role for the secretion of signalling molecules stimulating cell proliferation during the regeneration (Tran et al., 1998). The growth of the blastema then relies largely on the formation of a specialized wound epithelium called the Apical Epithelial Cap (AEC), its removal has an inhibitory effect throughout the regenerative process (Singer and Inoue, 1964; Stocum and Dearlove, 1972). Epidermal migration to cover the wound after amputation rapidly forms the AEC: at the beginning it is only a few cells thick, and over a few days it thickens to 8-15 cells. A remarkable fact is the lack of basement membrane under the AEC, and this constitutes the major difference

between wound healing in regenerating and non-regenerating systems (Neufeld, 1989). In the frog, which loses regenerative ability following metamorphosis, an AEC forms after amputation in the tadpole, but not in the adult limb. In adult frogs, closure of the wound after limb amputation is accomplished by the movement of the whole skin (dermis and epidermis) over the wound surface; this preclude direct contact between the epidermis and the underlying tissues, and results in the formation of a connective tissue scar (Carinato et al., 2000). In mammals, after skin injury the wounded area is quickly invaded by fibroblasts that within 48 hours start to deposit collagen and mucopolysaccharides, hindering the direct contact between the wound epithelium and underlying mesodermal tissues. Appearance of a partial blastema has been observed in amputated toes of adult mice in which formation of an AEC-like wound epithelium was induced by repeated surgical skin removal and NaCl treatment (Neufeld, 1980). In addition, some cases of regenerating fingertips have been observed in young children whose wound was not sutured after injury (Woolf and Boradbent, 1967). It appears that the basement membrane has an inhibitory effect on regeneration, and that signalling from the AEC to the mesenchyme is essential for the progression of regeneration and particularly for blastema outgrowth. The effect of the AEC can be largely replaced by application of proteins belonging to the family of fibroblast growth factors (FGFs), such as FGF-1, FGF-2, FGF-8 and FGF-10, (Albert et al., 1987; Boilly et al., 1991; Mullen et

al., 1996; Zenjari et al., 1996; Christen and Slack, 1997; Zenjari et al., 1997; Cannata et al., 2001; Yokoyama et al., 2001).

This proliferative stage of regeneration is also frequently nerve dependent. The nervous system has a pivotal role in the formation and morphogenesis of the regeneration blastema, but the presence of the nerve is required only during the early phase of regeneration. The presence of adequate innervation in the stump is a necessary condition for *Urodele* limb regeneration (*Salamander* and *Triturus*) and amphibian (*Triturus, Rana* and *Xenopus*) tail regeneration (Yntema, 1959a, 1959b; Simpson and Skirnyk, 1974). The blastema starts its formation only if the number of nerve fibers is at a supra-threshold level while the specificity of the innervation is not important (Hay and Fishman, 1961; Géraudie and Singer, 1978; Maden, 1979).

Transdifferentiation/metaplasia

When sufficient growth of the blastema has occurred, differentiation proceeds in a proximal to distal direction. An interesting aspect of the process that has yet to be completely investigated is the determination of the source of cells for the differentiated tissues in the regenerate. For the most part stump muscle contributes cells that eventually become regenerate muscle and connective tissue in the stump provides cells for its counterpart in the regenerate (Namenwirth, 1974; Dunis and Namenwirth, 1977; Kintner and Brockes, 1984; Fekete and Borckes, 1987, Echeverri et al., 2001). Nevertheless, there is

compelling and increasingly diverse evidence that conversion of one cell type into another, a process known as metaplasia or transdifferentiation, can occur in some cases. Recently the term transdifferentiation has become popular as alternative to metaplasia, particularly in the context to the ability of bone marrow stem cells to populate various other tissue types after grafting. In this context I used the term transdifferentiation in its original sense to refer to direct transformation of one differentiated cell type to another with or without cell division, and the term metaplasia to refer to any conversion of one differentiated tissue type to another regardless of pathway or mechanism (Tosh and Slack, 2002).

The first clear example of transdifferentiation came from studies of amphibian lens regeneration. In urodeles, removal of the lens from the eye stimulates the dorsal iris of pigmented retinal epithelium to regenerate (Colucci, 1892; Wolff, 1895) In anurans, is the new lens arises from the epithelium of the outer cornea inner layer (Freeman, 1963) In both cases these tissues de-differentiate, proliferate and transdifferentiate as a lens (Yamada, 1967; Brahma and van Doorenmaalen, 1968; Brahma and McDevitt, 1974; Yamada and McDevitt, 1974; McDevitt, 1982;). This example of transdifferentiation is very significant in regard to cell fate switching, because a specialized cell type like the pigmented retinal epithelium or the outer cornea epithelium are able to undergo an impressive change. The epithelial cells lose their cytoplasmic contents and the cell becomes reprogrammed to re-enter the cell cycle and to produce specific proteins of the

lens, the crystallins. Initially the cells de-differentiate and proliferate, forming an undifferentiated spherical mass of epithelial cells, the lens vesicle. In the following days the cells positioned deeply in the vesicle stop dividing and start the synthesis of the crystallins. As a result of the high rate of protein synthesis the cells accumulate the crystallins in the cytoplasm and become elongated into primary lens fibres. In the equatorial region the epithelial cells still surrounding the primary nucleus of the lens begin to express crystallins, become elongated and arrange themselves around the primary nucleus by forming the secondary fibres of the lens. At the same time the primary fibres lose the mitochondria and the nucleus and become fully differentiated lens cells. The continuous accumulation of secondary fibres is responsible for the growth of the regenerating lens. When the regeneration process is completed the regenerated lens will become independent from the original tissue and with its continuous growth will be functionally and structurally equivalent to the original lens (Brahma and McDevitt, 1974; McDevitt, 1982; McDevitt and Brahma, 1982; McDevitt and Brahma, 1990; McDevitt et al., 1997).

Regeneration territories

For complete regeneration, the presence of an adequate stimulus is not sufficient, but it is required that it acts on a competent "territory". In lower organisms (i.e. Hydra) every body parts has the potential to regenerate a complete individual (Tardent and Morgenthaler, 1966). With the higher grade of

structural complexity, the regenerating capacities have been progressively restricted to particular body areas with specific morphogenetic potentiality: so called "regeneration territories". Therefore, territories for fore limb, hind limb, tail can be shown to have the capacity to regenerate respectively just fore limb, hind limb and tail. The morphogenetic potential of a regenerating territory is due to a specific properties of its own cells and it is not necessarily determined by the particular position in the organism (Yamada, 1966). A very interesting aspect of regeneration is the retention in the regenerating part of the original polarity of the organism. Child (1941) explained this phenomenon by the theory of the metabolic gradient, and Slack (1985) by a comparison of properties between migratory cells and resident cells. In limb regeneration polarity seems to be controlled somewhat independently in the three anatomical axes: proximodistal, anteroposterior and dorsoventral. In this regard regenerating limbs and developing limbs are very similar (Bryant and Gardiner, 1992). During limb development, a region in the posterior part of the limb bud, the zone of polarizing activity (ZPA), plays a fundamental role in limb patterning. Signaling molecules normally expressing in the polarizing region, such as the secreted protein sonic hedgehog (*shh*), are expressed in the posterior part of the regeneration blastema. Also the *Hox* genes are active during limb development and are re-expressed following limb amputation. Their initial pattern of expression does not always reflect their developmental pattern, nevertheless the same pattern is established a few days later (i.e. 5' *HoxA* and *HoxD* during limb

regeneration) (Simon and Tabin, 1993). The cells of the blastema maintain the memory of their position of origin, since under normal condition only the missing part re-grows.

Epimorphic regeneration exhibits also activities that appear in the development of many cancers. The process by which the cancer cells spread from their origin locus of formation to secondary sites, metastasis, relies on cellular adhesion to extracellular matrix (ECM) and on matrix dissolution. Both of these behaviours facilitate cell migration, a pivotal mechanism of regeneration. The proliferating cells in the early phases of both regeneration and tumourigenesis express matrix metalloproteinases (MMPs) which selectively degrade ECM components (Saarialho-Kere et al., 1993; Yang and Bryant, 1994; Carinato et al., 2000; Gourevitch et al., 2003). Invasive or metastatic capability involves upregulation of protease genes, downregulation of protease inhibitor genes, and the conversion of the zymogen forms of protease into the active enzymes. Matrix-degrading proteases are characteristically cell surface proteins, anchored by a transmenbrane domain, that bind to specific proteases, receptors or integrins (Werb, 1997; Stetler-Steverson, 1999). In fact integrin expression changes also are evident in invasive or metastatic cells. The successful colonization of a new tissue by a "foreign" invading cells is due to the shifting of the integrins that favour the ECM present in normal epithelium to those that preferentially bind the degraded stromal components produced by extracellular proteases (Varner and Cheresh, 1996; Lukashev and Werb, 1998). Nevertheless there is still some

uncertainty about whether these receptors play a central role in the capability of tissue invasion or metastasis, due to the large number of integrin genes and to the even large number of heterodimeric receptors resulting from combinatorial expression of various α and β subunits (Aplin, et al. 1998; Giancotti and Rouslahti, 1999).

Vertebrate tail regeneration

The remarkable regenerative capability to replace lost appendages in adulthood is restricted to a few vertebrate classes, in particular to fish and amphibians. The word appendages is generally used to refer to a variety of stretched out structures such as limbs, fins and tail. Significant variations in regenerative ability are observed in different groups and even among different species within the same family. In some species the regenerated appendages are morphologically and structurally a complete and functional replica of the original, whereas in others they can be a replacment structure that is not fully functional and even lacking core tissues. Since the first experiment of Spallanzani (1768), urodele amphibians have been the most used models to investigate the regenerating processes in vertebrates, because of their high regenerative capacities, superior to all the other vertebrates,for ecample the capacity of larva and adult of *Tritutrus* to replace lost appendages, the anterior part of the jaw, large region of the central nervous system (CNS) and eye is well known. But among all vertebrates regenerative capability is more widespread during development.

Even mammals retain the capability to regenerate lost appendages during their foetal life (Wilson, et al. 1989; Muneoka and Sanson, 1992). The anuran amphibians also have considerable regenerating capacities, similar to urodeles, but limited to larval stages. Generally these abilities decrease gradually with the progress of development until they completely vanish after metamorphosis (Beattie et al., 1990; Filoni et al., 1997). In mammals, such as mouse, significant regenerative ability has been observed in the digit tips of the foetus and appears to be correlated with the expression of *Msx1* and *BMP* genes ((Han et al., 2003). Also the neonate can regenerate its digit tips, although not always perfectly; it has been reported that even young children can regenerate their last phalange, including the nail, that appears to be important for the induction of bone re-growth (Illingworth, 1974; Mohammad et al., 1999; Neufeld and Zhao, 1993; Polezhaev, 1980; Singer et al., 1987). The ability to regenerate the tail is observed not only in amphibians, but also in *Lacertine* reptiles and fish larvae, nevertheless the urodele amphibians are the only order that can completely re-grow the missing part (vertebrae, spinal cord and ganglia, metameric muscle), both in adult and larva. Like other regenerative processes, tail regeneration proceeds in three steps: a pre-blastematic phase, blastematic phase and morphogenetic phase, however there is an additional degree of complexity in comparison to limb regeneration, since the tail consists of more heterogeneous tissues, as it includes spinal cord and sensory ganglia. Even though limb regeneration involves the regrowth of nerves, the nerve integrity is important,

not essential (Cannata et al., 2001), for progression of regeneration. Through the nerves important grow-factors are probably transported into the damaged area.

1. Pre-blastematic phase: after transversal amputation of fifty per cent of the tail length, the amputated surface is covered by the wound healing epithelium from the skin epidermis within 24 hours. This cell layer becomes thicker than the stump epidermis by continuous cell migration from the epidermis and it is in direct contact with the underlying undifferentiated tissues, without dermis or basement membrane. A few days later the de-differentiation of muscle starts and cells of the ependymal canal proliferate by forming the "neural ampulla", bringing about the closure of the ependymal canal of the spinal cord.
2. Blastematic phase: the mesenchymal cells eventually derived from the de-differentiation of stump tissues accumulate under the wound healing epithelium forming the regenerative blastema. Following this, the proliferating cells of the neural ampulla differentiate and form a new spinal cord alongside the blastema.
3. Morphogenetic phase: growth of the blastema continues, and several days after amputation new tissue differentiation starts. The muscle cells aggregate again into myofibres then into muscle fascicles. The fusion of the myoblasts proceeds in a proximal-distal direction, reforming segmental muscle. The regenerating spinal cord contacts the distal tip

of the blastema, and reforms a structures similar to the original with the formation of new neurons (after the differentiation of neuroblasts from proliferating cells of the ependymal canal) and regenerating nerve axons. The new ganglia start to form laterally to the spinal cord and the epidermis becomes thin and acquires a dermis and basement membrane once again (Filoni, 1981).

The regenerative capability of the amphibian tail does not usually correlate with autotomy, that is the reflex by which the tail can be shed in response to dangerous situation along a preformed fracture plane. With the exception of plethodontid salamanders and the salamandrid *Chioglossa lusitanica*, tail autotomy does not occur in urodeles and not at all in anurans (Dinsmore, 1977; Wake and Dresner, 1967). The major histological events of the regeneration process have reported to be very similar in both limbs and tail, even if the complexity of the two structure is different. Indeed the studies reported rely largely on the experimental model of urodele limb regeneration, nevertheless in the last decade more interest has been attracted by the tail regenerating model. Shortly after injury, bleeding ceases and the epidermis at the margin of the wound migrates over the damaged stump tissues to form the wound healing epithelium. Concomitantly, cells lining the ependymal canal of the spinal cord migrate to seal off the cut end to stop leakage of the cerebrospinal fluid, moreover those cells proliferate by causing the enlargement of the ependymal canal resulting in the formation of a structure so called "neural ampulla"

(Stefanelli, 1951). Necrotic cells, tissue debris, blood cells and macrophages are observed beneath the wound epidermis. Cell division is rarely seen in the wound healing epithelium, and new cells for the thickening of the ectodermal apical cap (AEC) are provided by division in the more proximal regions. The other damaged tissues of the stump break down, releasing cells which possess none of the recognizable characteristic of their tissue of origin. These cells accumulate beneath the wound epidermis and form the blastema, undifferentiated mass of multipotent proliferating cells, which will give rise to new appendage. Nerve fibres injured at amputation undergo some retrograde degeneration, and then begin to grow back into the accumulation of cells at the tip. The lizard tail blastema, in addition to peripheral motor and sensory nerve fibres, contains the spinal cord with some descending motor fibres. These latter accompany the outgrowth of the ependymal cell layer lining the neural canal, which forms the ependymal tube ending with a spherical enlargement, the neural ampulla, just beneath the epidermis. The ependymal tube is a pseudostatified neuroepithelium that strongly resembles the neural tube of developing embryos and will give rise to the neurons and glia of the regenerated spinal cord (Fig.I10) (Stefanelli, 1951; Egar et al., 1970; Nordlander and Singer, 1978).

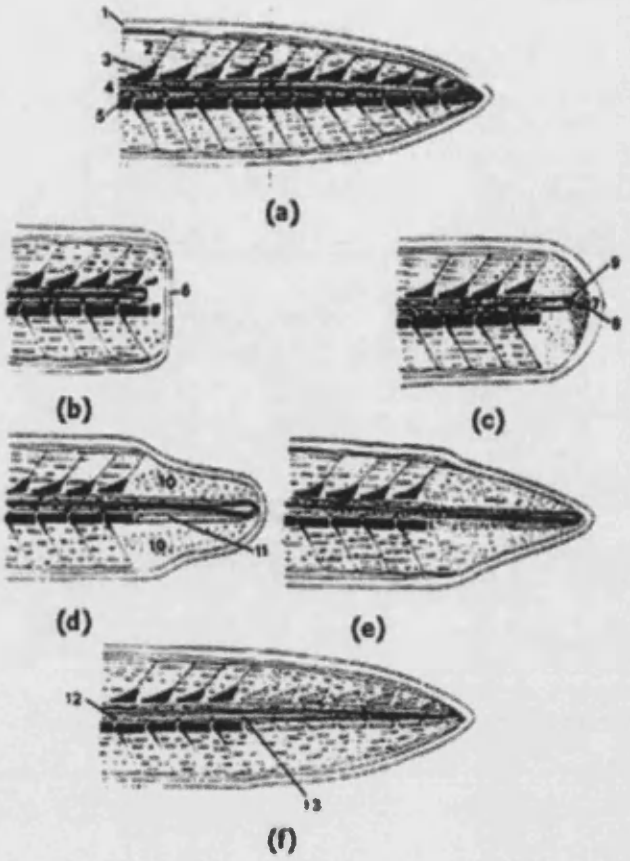


Fig.I10 Stages of regenerative process of the Urodels tail (sagittal section): **(a)** Normal structure and amputation level **(b)** Two days after amputation, preblastematic phase **(c)** After 10 days blastematic phase **(d)** and **(e)** After 15 and 20 days morphogenetic phase respectively **(f)** New tail structure after 45 days **1)** epidermis **2)** muscle **3)** neural arch **4)** spinal cord **5)** vertebral body **6)** wound healing epidermis **7)** apical cap **8)** blastema **9)** ampulla apicalis **10)** first muscle **11)** cartilage segment **12)** spinal ganglia of the stump 13) regenerating spinal ganglia (Filoni, 1981).

The blastema grows in size both by recruitment of cells from the stump and by mitosis. With enlargement the blastema rounds out and forms a visible outgrowth. At the time when a considerable amount of growth as has yet to

occur, differentiation of the cells of the blastema starts. In a short time the axial cartilage is laid down in continuity with the skeleton of the stump. Usually one week after amputation the blastema is well established, with the blastemal cell density highest beneath the growing ependymal tube. The tip of the regenerating tail is always the least differentiated part of the regenerate and contains the ependymal tube surrounded by blastema. As differentiation proceeds distally and laterally, the regenerate grows in length, and muscle and connective tissue are formed. A new appendage, limb or tail, in most of cases representing as much as 100 per cent of the amount of tissue which was lost, is produced and regeneration is complete (Roguski, 1953a; Roguski, 1953b; Bryant, 1970; Jabaley et al., 1976; Gardiner et al., 1986).

Anurans and *Xenopus laevis* tail regeneration

The tail in most anuran tadpoles (*Xenopus laevis, Rana clamitans, Rana temporaria, Rana pipiens, Hyla arborea*) has high regenerative capability. They are able to regenerate a complete and functional tail likethe original, nevertheless this ability is somewhat different from the urodele regeneration capacity. The anuran regenerated spinal cord has a more simple structure and lacks the spinal ganglia; the neural tissue does not seem to be able to develop much beyond the ependymal tube stage. The regeneration of the tail in anurans is characterised by a structural dependence on the notochord. It has been known for some time that in the absence of the notochord the regenerated tail in the

frog larva remains a stumpy and rounded structure without a posterior taper (Morgan and Davis, 1902; Marcucci, 1926). However, information on the effect of the absence of the notochord on the regenerative processes in tail tissues was scanty and contradictory. Marcucci (1926) amputated the tail through a piece of the notochord grafted in the dorsal fin and observed muscles growing alongside the regenerating graft. He concluded from this that the notochord had induced muscle formation. Twenty years later McCallion (1948), attempting to produce accessory tail in frog larvae, remarked that the notochord does not initiate regeneration in other tissue. Niazi (1966) performed notochord ablation adjacent to the amputation surface in *Rana clamitans* tadpoles to undertake specifically the study of the effect of the lack of a notochord on the initiation and progress of regeneration in other tissue . He definitely proved that in the absence of the notochord the regenerates do not acquire a normal tail-like shape, and remains a short and stumpy fin-like outgrowth. The regeneration of the spinal cord showed independence of the presence or absence of the notochord. Likewise, re-differentiation of the muscle began as usual even in the complete absence of the notochord and proceeded to give rise to striated muscle fibres. Similar results were obtained in lamprey larvae: when the stump notochord was destroyed after tail amputation and the resultant regenerate, although lacking a notochord, contained well formed and segmentally arranged myotomes. Nevertheless the regenerates were limp due to the absence of the notochord and thespinal cord deviated from its usual path, growing almost vertically downward (Niazi, 1964).

Therefore it is likely that for the anurans a suitable linear stretching of the tail is a necessary condition for regeneration to give rise to a tail of normal shape, size and internal organization. The growing notochord fulfils this condition and thus indirectly facilitates the normal progress of regeneration of other tissues. In amphibian embryos also, excision of the notochord results in great distortion of the spinal cord, vertebral cartilages and segmented muscles, which has been interpreted as a secondary effect of notochordectomy resulting in failure of the embryos to stretch normally (Horstadius, 1944; Kitchin, 1949). The considerable importance of the role that notochord plays during anuran tail regeneration is also evident in experiments performed in *Hyla arborea* (Stefanelli et al., 1950). After removal of a caudal spinal cord segment and the underlying piece of notochord, the two cut notochordal extremities regenerated towards each other and formed a single dorsal notochord growing in perpendicular direction to the tail. In these experimental conditions, the two cut extremities of the spinal cord each formed their own neural ampulla, grew perpendicularly and outward following the regenerating notochord and giving rise to a secondary regenerating tail (Fig.I11). Nevertheless the presence of the spinal cord is not a necessary condition for anuran tail regeneration; after caudal spinal cord removal from the stump, a new regenerated tail lacking of the spinal cord has been seen (Morgan and Davis, 1902).

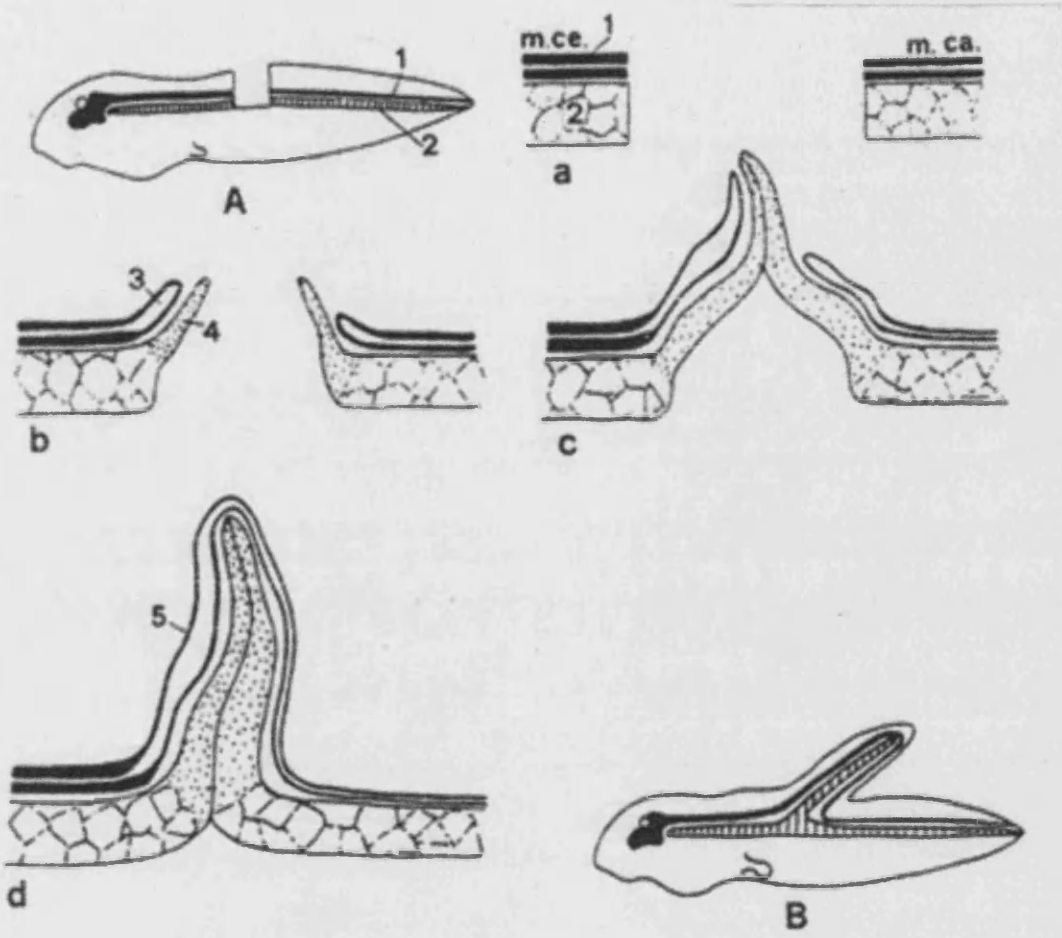


Fig.I11 Hypregeneration of *Hila arborea* tadpoles tail **(B)** after removal of spinal cord and notochord segment **(A) m.ca.)** caudal stump **m.ce.)** cephalic stump; **1)** spinal cord **2)** notochord **3)** ampulla apicalis **4)** regenerating notochord **5)** ependymal canal (Filoni, 1981).

In anuran tadpoles, also the formation of extra tails is independent of the spinal cord. Baita (1951), performing experiments in *Hyla arborea*, demonstrated that the formation of a secondary tail is led by the regeneration of the notochord. Removing a segment of caudal notochord from the ventral side of the tadpole

tail, he showed that the notochord, regenerating in a rostral-caudal direction, led the formation of a secondary tail directing outward from the original tail without the presence and effects of the spinal cord (Fig.I12). That again indicate that the notochord is needed as mechanic support during the tail regeneration process in order to drive and support the regenerate.

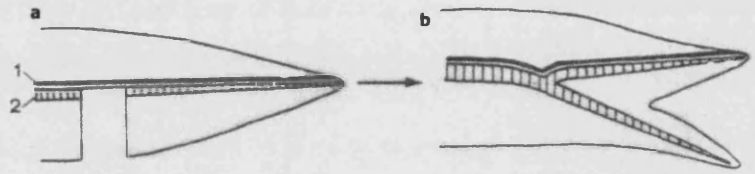


Fig.I12 Extra tail formation **(b)** in *Hyla arborea* tadpoles after removal of ventral segment of notochord **(a)**. The secondary tail was reformed without spinal cord **1)** spinal cord **2)** notochord (Filoni, 1981).

Although previous studies had already proved independence from the spinal cord during anuran tail regeneration, two further series of experiments were carried out. Rogusky (1954) performed spinal cord ablation, in *Xenopus laevis* and *Rana temporaria* tadpoles, by inserting several times a glass rod into the spinal canal in the cranial direction so as to destroy the end part of the spinal cord terminating at the amputation surface. Under these conditions regeneration

proceeded normaly unless the regenerate did not contain any spinal cord at all in which case the regeneration process was slower. Hauser et al. (1966) discovered a relationship between the shape of the tail regenerate and a neurogenic factor secreted in the cerebrospinal fluid. Hauser (1972) investigated the source of this factor ablating different region of the brain. Performing destruction of the whole telencephalon or of parts of the mesencephalic roof he did not see any effect in the tail regeneration, rather there was an effect following occlusion of the brain ventricles with 1 per cent agar (leaving the nervous structure intact), or electrocoagulation of the distal region of the diencephalic roof. Instead of being straight the regenerates were bent and distorted in a characteristic way (kinky). Histological examination of the diencephalic roof revealed the existence of an active secretory ependymal region the subcommissural organ (SCO). The SCO gives rise to the Reissner's fibre (RF), a mucopolysaccharide protein thread which passes along the whole length of the central canal. In the neural ampulla it enlarges into the massa caudalis, which gradually dissolves and releases the material through the ependymal wall of the ampulla into the surrounding tissue. Hence the SCO-RF system was thought well fit all the requirements for being the source and vehicle of the regeneration-promoting influence. But the experiments of selective ablation of the RF in the caudal part of the ependymal canal, or the destruction of the SCO, or of all the epithalamic structures, did not led to a complete inhibition of the tail regeneration. There was a shape distortion of the regenerate, but neither the the size nor the tissue composition of the

regenerating tail were affected. Recent work carried out in *Xenopus* showed that in tail regeneration the same genes are probably responsible as control tail development. Although some information of interest common to the regeneration-competent vertebrates is beginning to emerge there is still far to go in understanding the molecular events underlying tail regeneration. It is clear that there are changes in cell phenotype in the regenerating tail and that developmentally regulated molecules are re-expressed during tail regeneration, as seen above. Changes in the expression of intermediate filaments are observed in the regeneration tail bud and parallel those observed in the regenerating limb: in the blastema cells in both systems, vimentin and keratins 8 and 18 being co-expressed. Markers of neural progenitor cells, such as nestin and vimentin, which are not detected in normal spinal cord, are expressed in the regenerating ependymal tube (Maier and Miller, 1992; O'Hara et al., 1992; Walder et al., 2003). Very little is known about the signals that trigger and maintain the proliferative response of blastemal cells in the regenerating tail. Many growth factors and signalling pathways are likely to be implicated in these events. It has been shown that members of the FGF family genes and of the FGF receptor (FGFR) are regulated during limb regeneration. Recently (Zhang et al., 2000; 2002) showed the involvement of the FGF2 during spinal cord regeneration, in particular this growth factor may contribute to the proliferation of the cells of the ependymal canal. FGFRs are also expressed in the tail blastema cells, suggesting that FGF signalling is likely to play a role in the blastema as well as in ependymal

tube growth as shown in limb regeneration (Cannata et al., 2001; Yokoyama et al., 2001). Altogether many studies have done on anuran tail regeneration, functional studies to elucidate the role of individual genes and cell population are largely missing and present an exciting but significant challenge.

Urodele tail regeneration

Urodeles can regenerate their tails both as larvae and as adults; this feature is the reason why there have been so many studies on this amphibian family's tail regeneration. Regeneration of the urodele tail takes approximately 8 weeks, this relatively long time being due to the replacement of missing vertebrae. Though the basic sequence of events leading to tail regeneration is largely the same, there are some considerable differences between the tail regeneration processes of anurans and urodeles. Mainly those differences reside in the absence of notochord regeneration during urodele tail regeneration and in the remarkable role that the spinal cord plays in this process. In urodeles, unlike in larval anurans, the presence of the spinal cord and in particular of the grey matter in the stump, is necessary not only for regeneration of the cord itself, but also for regeneration of the whole tail. When the grey matter is mechanically removed, leaving only the white matter in place, neither the spinal cord nor any other tissue regenerates. Several experiments were performed where the tail spinal cord was removed and sciatic nerve deflected into it before tail amputation indicate that the nerves alone can not support tail regeneration (Kiortis et al.,

1959; Simpson and Skirnyk, 1974; Donaldson and Wilson, 1975; Nordlander and Singer, 1978). The spinal cord in urodeles plays a pivotal role, as the notochord in the anurans but acting in a different way: actively secreting factors for the progression of the tail tissue regeneration. The regenerating tail organises itself around the growing ependymal tube and there is evidence that this is required for cartilage differentiation. Experiments performed by grafting pieces of spinal cord beneath the tail skin, or into the muscle, results in the formation, from mesodermal tissues, of segmented cartilage just underneath the transplanted spinal cord (Kiortsis and Droin, 1961). Deviation of the spinal cord through the skin at the level of the dorsal musculature or dorsal fin will also induce the formation of a secondary tail schematised in Fig.I13 (Holtzer, 1959).

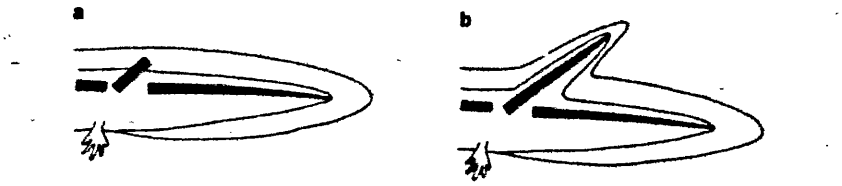


Fig.I13 Diagram of the promotion of an extra-tail in Urodele amphibians following the deviation trough dorsal direction of a spinal cord segment: **(a)** Deviation f the spinal cord; **(b)** Formation of the extra tail (Filoni, 1981).

Holtzer (1956), by rotating the spinal cord of axolotl larvae by 180° prior to tail amputation, showed the formation of a cartilage rod dorsally (up side down) above the rotated cord (Fig.I14).

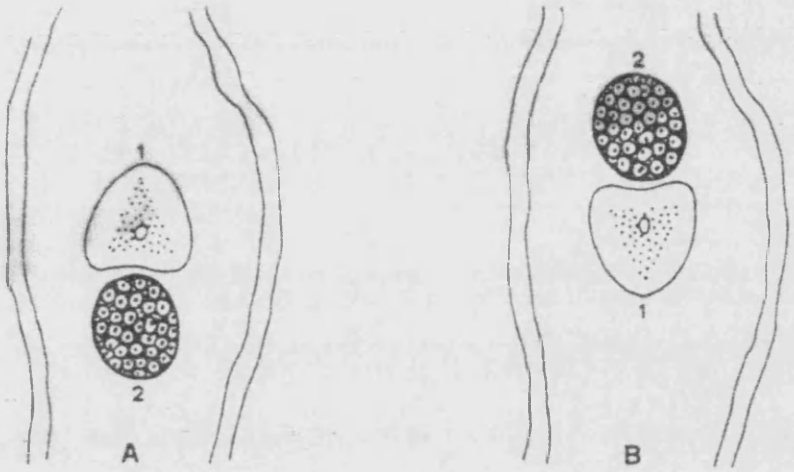


Fig.I14 Localization in the ventral half (motorial) of *Ambystoma* larvae spinal cord, of the factor that promotes cartilage formation: **(A)** Transverse section of regenerated tail after amputation **(B)** Transverse section of regenerated tail after rotation of caudal spinal cord and amputation at rotation level 1) spinal cord 2) cartilage segment (Filoni, 1981).

This suggests that the signals inducing differentiation of cartilage emanate from the ventral region of the stump spinal cord. This makes it likely to be attributable to the action of *Shh*. This gene is expressed in the floor of the neural tube and in the notochord; the fact that in the absence of the notochord the spinal cord is essential for urodele tail regeneration makes sense in terms of a requirement for Shh. Its expression plays a pivotal role in the formation of both

the myotome and the sclerotome during somite development: *Shh* promotes the formation of the sclerotome (*Shh* KO mice have severe defects in ribs development), and low concentrations have been found to promote myogenesis, an effect that is enhanced by the interaction and activity of some members of the Wnt family (*Wnt1* and *Wnt3*) expressed in the dorsal section of the neural tube (Martinez Arias and Stewart, 2002).

On the basis of morphological analysis it is currently believed that the regenerating spinal cord is formed by the radial glial cells that line the ependymal canal of the cord stump (Egar and Singer, 1972). During development of the sensory ganglia, the Schwann cells myelinating the peripheral nerves and melanocytes originate from the neural crest, a population of cells that emigrates from the dorsal aspect of the neuroectoderm. It has been clearly demonstrated both in larval anurans and in adult urodeles that the ependymal tube is required for regeneration of the spinal ganglia. Experiments performed in which spinal ganglia were removed while the spinal cord was left undamaged showed no regeneration of the spinal ganglia. On the other hand if all part of the spinal cord is ablated together with the ganglion, either for tail amputation or by complete damage of few spinal cord segments, a regenerated ganglion was formed (Bernardini, et al., 1992; Filoni et al., 1995). The regenerating cord seems to give rise also to Schwann cells and melanocytes. This is based on the experiments conducted on amputated spinal cord of the urodele *Pleurodeles waltl* labelled in the cut surface with a specific gene marker.

With regeneration proceeding Schwann cells and melanocytes showed labelling in the regenerate (Benraiss et al., 1996; 1997). It is not known, however, whether the cord is the sole source of these cell types.

Metaplasia

One of the most striking feature of urodele tail regeneration is the phenomenon of the adult tissue cell plasticity. Although this field has a long history of research, recent technical developments have made limb and tail regeneration an excellent system to understand how adult cells are transformed into progenitors that will rebuild the missing part. Cell tracking studies indicate that mature differentiated cells can dedifferentiate and radically change their identity during the process of regeneration. Two cell types were mainly used for cell tracing during urodele regeneration: skeletal muscle and spinal cord radial glia; they provided crucial evidence for the process called metaplasia by the creation of a multipotent progenitors that can re-differentiate into different tissues. Early histological studies indicated that muscles fibres might dedifferentiate by budding off mononucleate cells from syncytial muscle cells which then join in the formation of the blastema (Thorton, 1938; Hay, 1959; Hay and Fischman, 1961). By transplanting rhodamine-dextran labelled myotubes cultured *in vitro*, into the newt limb, Lo et al. (1993) showed mononucleated cells forming the blastema presenting the fluorescent signal of the rhodamine-dextran. The positive signal was still seen 4 weeks after amputation, when the tissues began to differentiate,

and the differentiation of positive cells was not limited to muscle fibres but labelled cells were also found infrequently in cartilage. Echeverri et al. (2001) reached similar results by pressure injection of a different fluorescent lineage tracer into tail muscle fibres of the axolotl. They followed the fate of endogenous myofibres in live tissue during tail regeneration and showed dedifferentiation of cells from injected muscle fibres into labelled mononucleate cells by 4 days post amputation, and subsequently proliferation and colonisation of the tail blastema. They estimated the percentage of dedifferentiated muscle cells populating the blastema corresponded to 17 per cent of the blastema tissue. The fate of the blatema cells could not be followed due to the dilution of the labelling signal thorough cell division. Therefore, it is not yet known if dedifferentiated muscle cells are capable to re-differentiate into other cell types apart from cartilage. Little is known also about the genetic events that drive these dramatic changes. Experiments performed using heterokaryons, multinucleated cells created by inducing fusion of two different cell types, demonstrated that the differentiated stated is actively maintained at the transcriptional level. Heterokaryons made fusing urodele and mammalian muscle suggested that the dedifferentiation programme is dominant, actively reversing the differentiation state (Blau, 1992; Velloso et al., 2000). Urodele myotubes are able to re-enter into the cell cycle S-phase during dedifferentiation in response to serum factors, whereas mouse myotubes do not response to those factors. In particular a pivotal role is played by thrombin, which activates an unidentified serum protein that initiates

dedifferentiation. As thrombin is normally activated following wounding this, may connect the initiation of de-differentiation to the response to the injury (Tanaka, et al., 1997; Simon and Brockes, 2002). It is also believed that the dephosphorylation of the rb protein is a critical intracellular step in the reentry to S phase, as mouse myotubes from Rb -/- animals do have this property. When urodele-mouse heterokaryos were made, the mouse nuclei were stimulated to re-enter S-phase of cell cycle, indicating that a cytoplasmic factor in the urodele myotube acts in "trans" over the mouse cell cycle block. The effects of *Msx1* during anuran tail and mouse digit tip regeneration have already been described. Odelberg et al. (2000) demonstrated that the expression of *Msx1* in mouse myotubes caused a small fraction to dedifferentiate into mononucleated cells. These cells were able to re-differentiate into chondrocytes, osteoblasts and adipocytes under appropriate inductive stimulus. These authors suggested that *Msx1* expression was sufficient to drive them back to a mesodermal precursor. Experiments on spinal cord cell-tracing during axolotl tail regeneration revealed that neural progenitors can even cross the boundaries between embryonic germ layers. It has been already shown that the radial glial cells lining the ependymal canal, proliferate to form the ependymal tube. These cells are probably equivalent to the neural progenitors of the embryonic and adult mammalian central nervous system (Doetsch, et al., 1999; Malatesta et al., 2000; Noctor et al., 2002). They derive from similar anatomical cell layers, and express similar markers such as glial fibrillary acidic protein (GFAP), that appears to be found in

both urodeles and mammals. Echeverri and Tanaka (2003) using a GFAP promoter driving the green fluorescent protein (GFP) followed cell stability of the radial glial cells of the ependymal canal during axolotl tail regeneration. They introduced the DNA by electroporation, using a DNA-filled microelectrode introduced into the lumen of the spinal cord through the cut end of the tail. GFP expression was under the control of the GFAP promoter via the GAL4-VP16 enhancer (Koster and Fraser, 2001; Echeverri and Tanaka, 2003). In this system the GFAP promoter drove the expression of GAL4-VP16 fusion protein, while GFP was driven by the GAL4UAS, the target sequence of the GAL4 protein. Inclusion of the GAL4-VP16 enhancer system ensured that GFP persisted, even if the transdifferentiation of those cells turned off the GFAP promoter, because high levels of the GAL4-VP16 protein initially produced the GFP remained expressed during tail regeneration. They followed at the single cell level the fate of the previously labelled radial glial cells and found that their descendants were not restricted to an ectodermal cell fate, the original germ layer of the neural progenitors. Although many labelled cells formed the expected neural cell types, they also contributed to regenerating mesodermal structures such as muscle and cartilage. These studies illustrate that radial glial cells originally derived from ectodermal tissue can switch lineage during tail regeneration and transdifferentiate in muscle cells, a cell type originatating from the mesoderm. In relation to these results there is at present not sufficient evidence to exclude definitively the involvement of cell fusion events during urodele tail regeneration.

A large series of reports has indicated that marrow derived cells were found to engraft as differentiated cells into multiple tissues after infusion of experimental animals of either whole bone marrow or one or more subpopulations from marrow. Also, several recent publications have described isolation of precursor cells for nonhematopoietic tissues from peripheral blood (Chesney and Bucala, 2000; Zvaifler et al., 2000; Kuznetsov et al., 2001). Furthermore those studies have suggested that bone marrow cells possess the capability to differentiate in different cell lineages such as liver cells, cardiomyocytes and neurons. Several groups have attributed this plasticity capacity to transdifferentiation; others, however, have suggested that cell fusion events could explain these results (Alvarez-Dolado et al., 2003). Although the contribution of cell fusion to the engraftment and differentiation of marrow derived cells observed *in vivo* experiments has been defined. The available data do not resolve questions such as: are the fused cells dead end products that disappear with time, or are they intermediates in the normal process of tissue repair? (Prockop et. al., 2003).

Although some results might be due to cell fusion, in general the cellular and molecular observations reveal that cell plasticity is a real process occurring in urodele tail regeneration and that tail injury is able to trigger reprogramming in a cell population regardless of the embryonic germ layers of origin.

Gene activity

The gene expression pattern during urodele tail regeneration is generally similar to the anuran. An unusual pattern of expression has been reported in urodeles for two genes: *dlx-3* and *Wnt-10*. *dlx-3* is a homologue of the *Drosophila* gene *distal-less*. It is detected both in regenerating muscle and spinal cord, whereas it does not appear to be expressed in the developing nervous system in other species. In the regenerating cord a strong signal is detected in the ventro-lateral region. Therefore, it has been proposed that within the regenerating cord *dlx-3* may label the cells with neural crest-like developmental potential which migrate along the developing ventral roots and give rise to the sensory ganglia; nevertheless its role is not completely understood (Mullen et al., 1996; Nicolas et al., 1996). The BMP pathway seems to be very important for the exact reconstruction of the lost tail tissue. Beck et al. (2001) showed the key role played by the elements of the *BMP-4* pathway during tail development: BMP receptor, *Smad5*, *Notch* and *Xhox3*. They showed that there are two different pathway starting from BMP: one proceeding through the canonical Smad cascade; the second one mediating by the *Notch* pathway. Ectopic expression of an activated form of *Smad5* in the posterior neural plate resulted in the formation of an extra tail-like structure with well formed somites, neural tube and fin. *Notch* or *Xhox3* stimulated the formation of an extra tail-like structure as well but lacking somites. Similarly in a more recent publication Beck et al. (2003) showed that the same pathway of *BMP-4* must be reactivated to the

starting of the tail regeneration process. They showed the stimulation of tail regeneration in the refractory period of the *Xenopus* larvae (st. 45-47 NF), by producing inducible transgenic embryos, under the normally silent heat shock promoter HSP70 (Bienz, 1984), expressing elements of the BMP or *Notch* signalling pathways after heat shock at 37°C. Stimulating the two different pathway they obtained complete regenerating tail with somites (BMP) or regenerating tail with neural tube and notochord but lacking somites (*Notch*). Therefore the gene functions during tail outgrowth seems to be necessary as well for the activation of tail regeneration. Moreover Beck speculated on *Msx1* as the main target of the BMP pathway. Expression of *Msx1* is directly activated by *BMP* signalling (Suzuki, et al. 1997), and *Msx1* can induce the dedifferentiation of mouse myotubes in culture from quiescent multinucleate cells to mononucleate, proliferating cells (Odelberg et al., 2000). They demonstrated that *Msx1* is actively transcribed in regenerating but not in non regenerating tail, and that *Msx1* expressing transgenic tadpoles, which normally would not regenerate after tail amputation, were able to regenerate a well patterned tail including myotomes. Thus *Msx1* is able to mediate all the effects of BMP. Furthermore using a truncated form of *Msx1* they did not obtain any stimuli for tail regeneration in non regenerating larvae. Even though mammals are not endowed with such regenerative abilities, the tips of the toes in mice and even humans (young children) can be regenerated only when amputated distally and the wound is impaired to heal (Illingworth, 1974; Han et al., 2003). An

interesting association with this digit regeneration is the expression of *Msx1*, already reported in mice. Therefore Goss has proposed that inhibition of blastema formation by dermal healing (as opposed to wound healing epithelium) seen in mammalian limb amputation could account for the loss of regenerative ability in mammals (Goss, 1980).

Lizard tail regeneration

The lizard tail is the only reptilian appendage that can regenerate. Only species that can undergo tail autotomy, however, appear to have such regenerative capability and there is total coincidence between autotomy fracture planes and regeneration-competent territories. In the lizard, unlike urodele and anuran amphibians, the regenerate is not a faithful copy of the lost structure. When lizards regenerate their tail following loss or injury, a simplified structure replaces the lost one. In particular the new structure lacks the segmented vertebral column which is replaced by a continuous unsegmented cartilaginous cylinder (Woodland, 1921; Quattrini, 1954). Numerous experiments on different species of lizards have documented the poor ability of the regenerating tail regarding mechanical activity (Werner, 1967; Bellairs and Bryant, 1985). Most of the reformed tail, since it is no longer articulated, is inefficient for active movement, but it carries on a function as a balancer and fat deposit organ. Its limited function also arises from the calcification of the regenerated cartilaginous cylinder, which occurs rapidly after tail loss (Alibardi and Sala, 1981). The

course of regeneration is as follows: in a short time after tail amputation the cells of the lesioned periosteum and the intervertebal cartilage proliferate and invade the forming blastema (Simpson, 1964; Alibardi and Sala, 1983). These chondrogenic cells organize themselves around the regenerating spinal cord, forming a conical structure with the base contacting the original vertebrae and the apex tapering toward the apical blastema where they form a precartilaginous anlage (Cox, 1969). The procartilaginous cells are oriented perpendicularly to the regenerating spinal cord and they actively proliferate. Following differentiation, the chondrocytes mature in a proximo-distal direction into hypertrophic hyaline chondrocytes. The mature cartilaginous tube of the regenerated tail is quite stiff and the inner and outer surfaces of the cartilaginous cylinder become calcified; in very old regenerated tails even ossification may take place (Fig.I15). An exception, however, seem to be some species of gecko which display a regenerated tail that is surprisingly active and flexible and possesses a prehensile function due to its ability to curl up. In this case the regenerated tail never becomes stiff because of the formation of elastic cartilage in which no calcification occurs (Alibardi and Meyer-Rochow, 1989). Regenerated chondrocytes probably originate from elastic precursor chondrocytes derived from intervertebral disc or perichondrium. It was shown that the intervertebral disc displays mixed characteristics of fibrous and elastic cartilage with oriented thin and elastic fibres. Detailed studies on the cartilage of different reptilian species have shown a precise relationship among hyaline cartilage, amount of

intercellular matrix material, speed rate of regeneration and functionality of the regenerated tail.

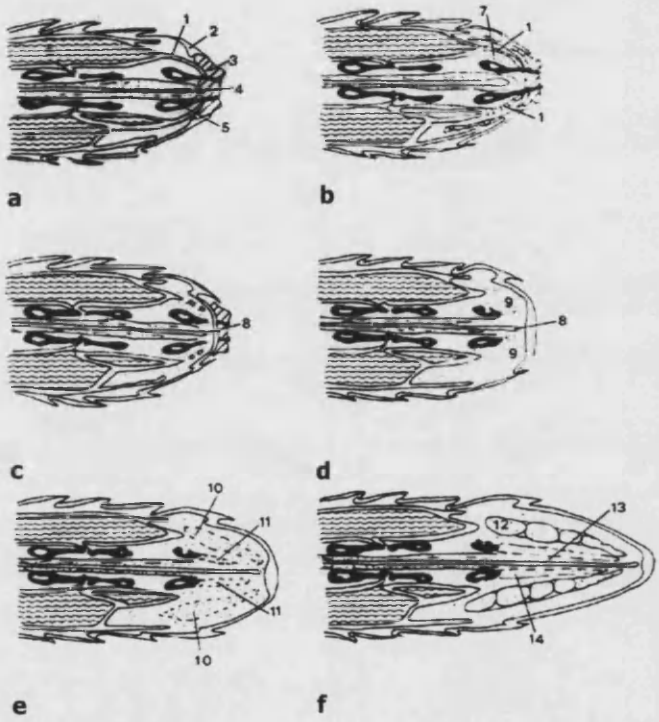


Fig.I15 Schematic time course of the tail regenerating process in lizard (from longitudinal sections). **(a)** At 24 hours after autotomy the damaged surface is covered by blood cloth. **(b-c)** Respectively at 4 and 6 days from tail removal, the wound healing epidermis starts to be formed and there is the dedifferentiation of the fat tissue and vertebrae. Formation of the neural ampulla. **(d)** At 8 days starts the blastema formation beneath the wound healing epidermis. **(e)** After 10 days from the amputation the neural ampulla gives rise to the ependymal tube. **(f)** After 15 days starts the formation of the cartilage rod and the metamerization of the regenerating mucles. **1)** connective tissue; **2)** epidermis; **3)** blood cloth; **4)** spinal cord; **5)** vertebrae; **6)** muscles; **7)** fat tissue; **8)** neural ampulla; **9)** blastema; **10)** early regenerating muscles; **11)** early cartilage rod; **12)** differentiated muscles; **13)** ependymal tube; **14)** mature cartilage rod (FIloni, 1981).

It was shown that there is a progressive passage from typical hyaline cartilage, rich in matrix matter, where the tail is of little mechanical importance, to a hyaline cartilage poor in intercellular matrix where the tail is considerably more active. Finally in *Holoplodactylus* a real elastic cartilage is present, reflecting the need of a very active and prehensile tail in this species. Moreover a cartilage poor in intercellular substance is present in species with long regenerated tails and a fast rate of regeneration, while short regenerated tails with low rates of regeneration have typical hyaline cartilage. Scanty intercellular matrix material and turgid cells improve the flexibility of the tail: it can thus become an efficient organ for climbing, carry out prehensile functions and act as a balancing organ for arboreal life (Anderson, 1964; Alibardi and Meyer-Rochow, 1989).

Most of the rapidly regenerating cartilage is derived from interstitial growth and not from the appositional growth of new chondroblasts from the outer and inner perichondria (Shah and Chakko, 1968). The homogeneous cell proliferation and the presence of a continuous perichondrium covering the forming cartilaginous tube is not favourable for the segmentation of vertebral anlagen. In fact, during caudal regeneration in the newt, the formation of segmented vertebrae from an initially compacted rod of cartilage is associated with the interruption of the perichondrium into segmented regions called "intercentra", the future intervertebral discs (Holtzer et al., 1955; Alibardi and Sala, 1981)..

Alibardi (1995) showed that the reformation of the segmental organization in the regenerating lizard tail muscles is due to the formation in the tail apical region of

pro-muscle aggregates. Cells characterised by different potentiality, myoblasts and fibroblasts, are mixed in the tail tip; following their joining in pro-muscle aggregates, because of different cell-cell affinities within this structure, the myoblasts recognise each other and start to fuse. The myogenic cells are programmed to segregate from the fibroblasts when aggregates of 4-8 myoblasts are fused into myotubes, representing the initial length of the segmented myotomes. Therefore the early myotubes, that will incorporate new myoblasts for further growth, arise from an event of cell fusion among a fixed number of myoblasts. The non-fusing fibroblastic cells simply remain around the myotubes, mostly close to their ends forming the connective tissue myosepta that delineate muscle segmentation. This morphogenetic mechanism appears considerably different from that of other vertebrates. In *Xenopus*, during development of the myotomes, long mononucleated myoblasts first appear and then extend over the entire length of the myotomes. Later, these mononucleated myofibers become multinucleated cellular syncytia by addition of new cells at their ends. The possibility of nuclear division or nuclear amitotic fragmentation has also been proposed (Muntz, 1975; Boudjelida and Muntz, 1987). Despite these differences, the formation of myotubes between the myosepta is of fundamental importance for muscle segmentation; in fact to maintain this ordered proximo-distal organisation, most of the new myoblasts are added near the tip of the myotubes. A peculiar feature of lizard tail regeneration is the effect that the method of tail loss has on the regenerating process. After experimental

tail amputation, the damaged muscles are repaired by resident muscle satellite cells. In contrast, natural or induced tail autoamputation is followed by muscle regeneration, mostly derived from the migration of myogenic cells from the connective intermuscular septum into the blastema. In autotomy the tail splits along preformed breaking planes, and thus avoids injury to muscle fibres. Nevertheless in both cases there is no evidence for any contribution to the regenerative blastema by dedifferentiation of cells from stump muscle fibres (Kahn and Simpson, 1974; Simpson and Bayne, 1979; Bellairs and Bryant, 1985). As discussed above, the vertebrae do not regenerate and the cartilaginous tube acts as a replacement for the lost vertebrae. The cylinder of cartilage wraps around the regenerating "spinal cord", a structure much simplified from the original: containing only ependymal cells and descending axons, whose elongation is driven by a channel formed by the ependymal cells processes, as in the newt tail. The regenerate does not contain differentiated and mature neurons, the axons innervating the regenerated tail originate mainly from spinal neurons rostral to the amputation level. As in urodeles, lizard tail regeneration depends on the spinal cord and in particular on the ependymal cells. Ependymal tubes of regenerated tails grafted obliquely into the dorsal tail muscle induce growth of additional tails, and tail hyper-regeneration (multi-tip tail forming) occurs following the formation of secondary ependymal tubes after incomplete autotomy (Simpson, 1964; Goss, 1969). In lizards, embryonic tails regenerate during the week preceding hatching in a similar manner to the adult, but at

earlier developmental stages regeneration is less complete. Although an ependymal tube is present and mesenchymal cells accumulate beneath a thickened wound epidermis, differentiation is not observed. It appears therefore that the tail tissues have to reach a certain degree of maturity to acquire maximal regenerative capability. This may again correlate with their ability to autotomize the tail and indicate a strong adaptive component to the events of lizard tail regeneration. At early developmetal stages the embryo is within the oviduct, and therefore protected from predators. Therefore, there would be no adaptive need for tail regeneration at this stage. This suggests that some examples of regeneration may represent genuinely new mechanisms and not just the recapitulation of developmental process. It also shows that the highest regenerative capability is not always associated with early stages of development (Bryant and Bellaris, 1970; Ferretti, 2001).

Aim of the research

For a century anuran regeneration, including *Xenopus laevis* tail regeneration has been investigated with regard to morphogenesis and the specific aspect of tissue ablation. Nevertheless the destiny of cell populations during the tail regenerative process has not attracted particular attention. Two main questions are addressed in this work:

- Whether the regenerate originates from mature differentiated cells or from undifferentiated "reserve" cells?
- Does each tissue give rise to just its own cell type in the regenerate, or can it switch fate and differentiate to a different cell type (metaplasia)?

Several other studies have been carried out regarding metaplasia during the regenerative process, but those researches were mainly focused on urodele amphibians. The capability of cell plasticity in the urodele axolotl spinal cord has already been mentioned (Echeverri and Tanaka, 2003). Several years earlier Namenwirth (1974) and Dunis and Namenwirth (1977) investigated the transdifferentiation of triploid tissue grafted in the axolotl limb and caused to regenerate by amputation through the graft region. Even though these experiments permitted a certain degree of cell-tracking they did not allow an in vivo analysis of the regenerative process. An in vivo specific tissue label is necessary for a direct and complete analysis of the cell stability of the different tissues involved in the events of regeneration, and this was done by Lo et al 1993). These studies on urodeles all show some degree of metaplasia.

The exploration of cell type stability is of some interest for further application in cell therapy and in general in the biomedical field, given the recent reports regarding tissue repair in mammals. Cohnheim in the middle of the 19th century suggested that all the cells involved in wound repair come form the blood stream, then from the bone marrow. But this was ruled out by a number of early experiments (Ross et al., 1970). Most studies on wound repair were focused on cells resident in tissues such as pericytes, seen to proliferate during the regenerative process in most tissues (Prockop, 1997). The interest in resident stem cells has grown gradually with the increase of publications reporting plasticity of stem cells like in several tissues: muscle, liver and brain (LaBarge and Blau, 2002; Campagnoli et al., 2001; Taupin and Gage, 2002). Some recent observations have supported Cohnheim's hypothesis indicating the replenishment of resident stem cells by stem cells for nonhematopoietic tissues found in the marrow. The results of those experiments performed to address the topic of the plasticity of nonhematopoietic cells presented some discrepancies, due to a number of critical variables: differences in the subpopulations of the cells used, stability of the genes used to mark the donor cells, level of engraftment of marrow-derived cells into non haematopoietic tissue, degree of tissue injury that promotes engraftment. Furthermore several reports indicate that some of the plasticity of adult stem cells can be attributed to cell fusion and not to transdifferentiation (Ying et al., 2002; Terada et al., 2002; Wang, 2003). Cell fusion was observed when adult stem cells from brain or unfractionated

mononucleated cells from marrow were added to cultured embryonic stem cells. The resulting tetraploid cells exhibited full pluripotentiality, including multilineage contribution to chimeras. Bone marrow derived hepatocytes were serially transplanted into a transgenic mice model for tyrosinemia. The resulting hepatocytes rescuing the mice arose from cell fusion and not from differentiation of marrow derived hepatocytes (Vassilopoulos, et al., 2003). This phenomenon of somatic fusion raises a fundamental question of whether the fusion between hematopoietic cells and brain, liver and heart cells has a physiological role in the development or maintenance of these organs. Experiments performed by Alvarez-Dolado et al. (2003) suggest that cell fusion may be the mechanism by which these cells become multinucleated or polyploid. Genetic material derived from blood cells may contribute through cell fusion to the survival and function of cells in different organs. At the moment it is difficult to resolve all of the apparent discrepancies in observations on the mechanism of tissue repair by bone marrow derived cells; nevertheless in both cases stem cells could be used in reparative cell therapy as reported by Sampaolesi et al. (2003). They demonstrate that intra-arterial delivery of wild type mesoangioblasts, a class of vessel associated stem cells, corrects morphologically and functionally the dystrophic phenotype in adult immunocompetent α-sarcoglycan (α–SG) null mice, a model organism for limb-girdle muscular dystrophy. The mesoangioblasts were isolated from juvenile dystrophic mice and trasduced with a lentiviral vector expressing α–SG in order to reconstitute normal skeletal muscle.

Mesoangioblasts showed the ability to cross the endothelium and migrate extensively in the tissue interstitium, where they were recruited by regenerating muscle fibres, thus reconstituting the dystrophin-glycoprotein complex. Although this is also the fate of blood-borne progenitors from the bone marrow, nevertheless the frequency of this event is too low to result in noticeable amelioration of the dystrophic phenotype (Ferrari, et al., 1998; Gussoni, et al., 1999; Ferrari et al., 2001). The first goal of this research was to establish a good *in vivo* tissue-specific cell-tracing method by using a transgenic technique in order to label single tissues with the expression of GFP. The core tissues of the tail: neural tube, notochord and muscle, were each targeted in order to trace their fate during regeneration. For this tissue labelling a new grafting technique was introduced by transplanting orthopically a segment of neural tube, notochord or presomite plate from the presumptive tail region of donor embryos, expressing GFP in whole embryo, to the same place of normal hosts at the same stage (stages 13 NF and 17 NF). To drive GFP expression ubiquitously, in the entire embryo, a good ubiquitous promoter was needed. Furthermore the promoter chosen must retain the expression of the GFP until the complete process of regeneration has occurred (stage 52/53 NF) and it must keep expressing the GFP in the regenerating tissue, in order to follow completely the process and allow a full cell-tracing. The promoters examined were: EF1α (Elongator Factor 1α promoter), CSKA (Cytoskeletal Actin promoter) and CMV (Simian Cytomegalovirus promoter). The choice was made on the basis of the

strength of the fluorescent signal and the persistence of the signal during development and regeneration. Because of the weaker activity of the EF1α promoter and the loss of activity of the CSKA promoter during development, the CMV promoter was chosen for this study. It was found that a single specifically labelled tissue, expressing GFP constitutively under CMV promoting activity, can be easily traced during tail regeneration. The single tail tissue transplants were analyzed to verify that the grafting technique was really precise and the GFP expression was limited to the targeted tissue. Grafted embryos and tadpoles were sectioned and immunostained with a commercial antibody against GFP in order to verify the exact localization of the fluorescent signal deriving from the transplanted tissue. Once the specificity of the graft technique had been proved the research proceeded by ato analyse the stability of the fluorescent signal in the tissue target during tail regeneration. After tail removal the localization of the GFP expression can be followed *in vivo*, by anesthetizing the tadpoles and examining them under a stereo microscope equipped with a UV lamp. This method was used to find out whether the GFP positive cells were stable, that is whether the fluorescent signal is retained in the same original transplanted tissue or is expressed in a different tail tissue, which would indicate the occurrence of metaplasia. During this study not just the problem of cell stability has been addressed, but also the origin of regenerating tissues. Apart from the urodele regenerating neural tube (Stefanelli, 1951)there was not previously clear evidence regarding the origin of the tissues of the tail

regenerate. Experiments with the injection of the thymidine analogue BrdU (5-bromo-2'deoxyurudine) and immunostaining against BrdU and PCNA (proliferating cell nuclear antigen), on serial sections of regenerating tails, were performed to analyse the cellular structure of the tail regeneration bud and the cell populations contributing to the regeneration of each of the single tissues. This study provides evidence to fill the gap in out knowledge of the origin of the regenerating tail tissues, especially regarding *Xenopus* notochord and neural tube regeneration. It was found more difficult to address the question of the origin of the regenerating muscle, because the labelling of the regenerating muscle fibres occured only if the presomite mesoderm (PSM) graft was performed at later stage (stage 17 NF). Experiments were performed to exclude the involvement of other tissues in tail muscle regeneration (transdifferentiation). Thus skin grafts, at larval stage (stage 48 NF), were performed in order to investigate if dermis cells could dedifferentiate and give rise to myofibres. Moreover DiI-LDL (low density lipoprotein) was injected in the *Xenopus* heart (Levine et al., 2003) in order to label endothelial cells of the blood vessels and then to analyse if pericytes (the outer cells of capillaries) could contribute to the regenerating tail muscle. Finally the behaviour of the *Xenopus* muscle fibres during regeneration was investigated: to find whether they behave like urodeles regenerating muscles (dedifferentiation and budding from the uninjured fibres) or like mammals (regeneration by muscle satellite cells proliferation and differentiation). For this purpose, Pax-7 monoclonal antibody was used as a

muscle satellite cells marker in order to analyse if Pax-7 positive cells contributed to the regenerated muscles. Thus double immunostaining against GFP and Pax-7 was performed to investigate the co-localization of green fluorescent positive cells and Pax-7 positive cells, and the contribution of those cells to the tail muscle regeneration.

II- Materials and methods

Embryos and tadpoles

Xenopus laevis embryos and larvae were obtained by standard procedures and staged according to the Nieuwkoop and Faber (NF) tables (1967). Embryos were dejelled with 2% cysteine (BDH) pH 7.9, and then cultured in NAM/10 with 5mM Hepes (NAM, Normal Amphibian Medium: 110mM NaCl, 2mM KCl, 1mM $Ca(NO_3)_2$, 1mM $MgSO_4$, 0.1 mM Na_2EDTA) at 24°C, in Petri dishes coated with 1.5% (w/v) noble agar. They were fed on nettle powder from one week and at about stage 47 transferred to a recirculating aquarium at 25°C. Conditions in this aquarium are such that the ammonia level is kept near zero and the pH is around 8.0. They are fed 3 times per week on tadpole diet (Blades Biological, Redbridge, UK).

Plasmid construction

The experiments for the production of GFP transgenic embryos were performed by using cDNA from several plasmid constructs:

pCD-CMV/nucGFP2: nucGFP2 was excised from CS2/nucGFP2 (a kind gift from E. Amaya) with BamHI and XbaI and cloned into Bam/Xba sites of pcDNA3 (Invitrogen). It was linearized with SmaI and diluted to 500 ng/μl final concentration for the integration in the sperm nuclei.

pCSKA-nucGFP2: nucGFP2 was excised from pCD-CMV/nucGFP2 with HindIII and XbaI blunted (Roche Klenow fragment) and cloned into HindIII/SmaI

sites of pCSKA (a kind gift from R. Harland). It was linearized with NotI and diluted to 500 ng/μl final concentration for integration in the sperm nuclei.

XeEF1α-nucGFP2: nucGFP2 was excised from pCD/nucGFP2 with BamHI and NotI and cloned into BamHI/NotI sites of XeGFP (a kind gift from G. Golling). It was linearized with NotI and diluted to 500 ng/μl final concentration for integration in the sperm nuclei.

NβTub-GFP and pCarGFP (kind gifts of E. Amaya) were grown in *E. coli* bacterial cells, linearized with NotI and diluted to the final concentration of 500 ng/μl to use for the transgenic technique.

pColII-nucGFP2: nucGFP2 was excised from pCS2/nucGFP2 with HindIII and Nsi both blunted (Roche Klenow fragment) and cloned in pKN/ColII (kind gift of Y. Yamada) and blunt cut with NotI. It was linearized with NotI, and diluted to 500 ng/μl final concentration for the production of transgenic embryos.

For the Cre-Lox system two different constructs were used together: promoter-Cre (i.e. Cre recombinase driven by the cardiac actin, muscle-specific, promoter) and pcDNA3/CMV-lox-STOP-lox-GFP (i.e. behaves like CMV-GFP following excision of the STOP sequence). pCarCre was made by excising the Cre+polyA addition sequence from pCS2Cre (a kind gift from D. Werdien) with HindIII and Not1, and cloning into pCar, after removal of GFP+polyA with the same enzymes. pCarCre was linearized with Not1I and diluted to a final concentration of 500 ng/μl. The construction of pcDNA3/CMV-lox-STOP-lox-GFP was made by substituting nucGFP2 by loxP-tpA-loxP-nucGFP from pCS2/CMV-lox-tpA-lox-nucGFP (made by M. Horb in the

lab). pcDNA3/CMV-nucGFP2 was cut with Xba and blunted with Klenow (Roche), then the nucGFP2 was excised with HindIII. The loxP-tpA-loxP-nucGFP was removed with Xba1 (blunted with Klenow) and HindIII. The loxP-tpA-loxP sequence came from pGK/neo-tpA-lox2 (a kind gift from P. Soriano) and the tpA has numerous stop codons to prevent translation. pcDNA3/CMV-lox-STOP-lox-nucGFP was linearized with SmaI and diluted to 500 ng/μl final concentration. All the plasmid constructs described above were grown in XL-1-blue chemically competent *E. coli* in Luria Broth (10 gr Tryptone, 5 gr Yeast Extract, 5 gr NaCl, 10 mM Tris pH 7.5 in 1lt H_2O) plus Ampicillin (Sigma) at the final concentration of 100 μg/ml. Plasmid DNA was extracted with the Wizard Plus Maxiprep kit (Promega) based on alkaline lysis of bacterial cells.

Transgenesis

The protocols for *Xenopus* transgenesis, based on the Kroll and Amaya method (Kroll and Amaya, 1996; Amaya and Kroll, 1999), involves the following steps: (a) incubation of sperm nuclei with linearized DNA (500 ng), (b) addition of high-speed interphase egg extract to the sperm nuclei and plasmid mixture to promote partial decondensation of sperm chromatin, (c) injection of one nucleus into an unfertilized egg in a 5-15 nl volume.

- Sperm preparation

The sperm preparation was made by following the standard protocol of Murray (1991).

A male was anaesthetized in 1% Benzocaine (w/v) (Sigma Ethyl 4-aminobenzoate) in water for 20 minutes. The ventral body wall and

musculature were cut and the yellow fat bodies were lifted to isolate the two testes which are attached to the fat bodies, one on each side of the midline. The testes were removed with dissecting scissors and placed in cold 1X MMR (MMR, Marc's modified Ringers: 1M NaCl, 20mM KCl, 20mM $MgSO_4$, 20mM $CaCl_2$, 5mM Hepes, 1mM EDTA pH 7.8). In a 35mm culture dish they were macerated with a pair of forceps and resuspended in 2 ml of 1X NPB (NPB, Nuclear Preparation Buffer: 250mM sucrose, 15mM hepes, 1mM EDTA, 0.25mM spermidine (Roche), 0.1 mM spermine (Roche), 0.5 mM DTT) by gentling pipetting the solution with cut tips (3mm in diameter). The sperm suspension is squirted through two-four thicknesses of gauze placed into a funnel and the solution collected into a 15 ml tube, then centrifuged at 3.000 rpm for 10 min at 4°C and the pellet resuspended in 1 ml NPB. Once the suspension was warmed (room temperature), 50 μl of 10 mg/ml lysolecithin (Sigma L-α-Lysophosphatidylcholine from egg yolk) were added for lysis of the sperm outer membrane. After 5 min of incubation were added 10 ml of cold 1XNPB + 3%BSA (w/v) with protease inhibitors: 1:1000 dilution of leupeptin (Sigma leupeptin hemisulphonate) and PMSF (Sigma phenylmethanesulphonyl fluoride) stock solution to the suspension and it was centrifuged at 3.000 rpm 10 min at 4°C. The supernatant was decanted and the pellet resuspended in 5 ml cold 1XNPB + 0.3%BSA (w/v) (without protease inhibitor) and was centrifuged again as before. The pellet was resuspended in 500 μl 1XNPB + 0.3% (w/v) BSA + 30% (w/v) glycerol (Sperm Storage Buffer) and kept at –20°C in aliquots. By using a Neubauer

chamber the sperm was counted (typically 75 –125 X10e4 cells/μl) and for the injection into the unfertilized eggs 300 sperm/μl were used.

(b) Cytoplasmic egg extract prep

12 Adult X. *laevis* female were primed 4 days prior to HCG injection by injecting 50U of PMSG (Sigma pregnant mare serum gonadotropin) into the dorsal lymph sac. The evening before egg extract preparation they were injected with 500U HCG (Intervet human chorionic gonadotropin). The frogs were then placed at 15-18°C (12-14 hr). The next day the eggs were gently manually expelled from each frog into a large dish of 1XNAM. As much NAM as possible was removed and the eggs were dejelled in 2% cysteine (w/v) (BDH) and washed in XB (XB, Extract Buffer: 0.1M KCl, 1mM $MgCl_2$, 0.1 mM $CaCl_2$, 50mM Sucrose, 10mM Hepes pH 7.7) and in CSF-XB (CSF-XB, Crude Cytostatic Factor-XB: XB + 5mM EGTA pH 7.7) with protease inhibitor (leupeptin, chymostatin and pepstatin [Roche]). Using a wide-bore pasteur pipette, the eggs were transferred into Beckman ultraclear tubes and as much CSF-XB as possible removed and replaced with 1ml of Versilube F50. Then they were centrifuged 60 seconds at 1000 rpm (150 g) and then 30 sec at 2000 rpm (600 g). The excess of CSF-XB was removed (the inverted meniscus between the Versilube and displaced CSF-XB is easily visible) and replaced with 2 ml of Versilube. The eggs were centrifuged again 10 min 10.000 rpm at 2°C, in a swinging bucket rotor, to separate the three layers: lipid (top), cytoplasm (centre) and yolk (bottom). The cytoplasmic layer was collected with an 18 gauge needle by inserting the needle at the base of cytoplamic

layer and withdrawing slowly. The suspension was transferred in a fresh Beckman tube on ice, protease inhibitor was added to the isolated cytoplasm and was centrifuged for an additional 10 min at 10.000 rpm, again in a swinging bucket rotor. Then $1/20^{th}$ volume of ATP-regenerating system (Energy Mix: 150mM creatine phosphate (Roche), 20mM ATP (Roche), 20mM $MgCl_2$) was added and the clarified cytoplasm was transferred into TL100.3 tubes (Beckman thick wall polycarbonate tubes). $CaCl_2$ was added to a final concentration of 0.4 mM (to inactivate CSF and push the extract into interphase) and incubated 15 min at room temperature. The cytoplasm, after incubation, was centrifuged in the ultracentrifuge (Beckman TL-100) at 70.000 rpm for1 hr 30 min at 4°C. The cytoplasm was fractionated in four layers, top to bottom: lipid, cytosol, membranes/mitochondria and glycogen/ribosomes. The cytosol layer was removed by inserting a syringe into the top of the tube through the lipid layer and transferred to fresh TL-100 tubes and spun again at 70.000 rpm for 20 min at 4°C. 10 μl aliquots of the high speed cytosol supernatant were made, quick frozen in dry-ice and stored at –80°C.

(c) Transgenesis by sperm nuclear injection into unfertilized eggs

Prior to the injection, agarose-coated injection dishes and transplantation needles were made. 1.5% (w/v) Agarose in MilliQ water was poured into 35 mm Petri dishes, and before it had solidified a template (a rectangular square of plastic) was laid onto it and once solidified stored a 4°C for ready use. The needles were prepared by using borosilicate glass capillaries of a 400 μm diameter (Clark Electromedical Instruments). One was placed in a horizontal

needle puller (Sutter Instrument P-97 Flaming/Brown Micropipette Puller) and successively were clipped with a forceps to produce a bevelled tip of 60-80 μm diameter using a dissecting microscope micrometer for measurement.

The agarose-coated injection dishes were filled with 1X NAM + 6% (w/v) Ficoll (Sigma Ficoll 400), meantime the eggs were collected from the frogs (previously described) and dejelly in dejelly Buffer with fresh addition of DTT (16 mM final). The sperm reaction mix was made: 4 μl of sperm stock (4 X 10e5 nuclei) was mixed with linearized DNA (250-500 ng) in SSB, mixed well by gentle pipetting (using a clipped tip to avoid shearing the DNA) and incubated for 5 min at room temperature. While the sperm was swelling in the reaction mixture, the healthiest eggs were drawn into a wide-bore pasteur pipette and transferred to the square space in the injection dishes. After 5 min incubation, the egg extract (defrosted at the moment of use) was added to the sperm reaction mix and the new mixture was incubated for 10 min at room temperature. Then the mix was diluted 50-100 fold in MSDB (MSDB, Modified Sperm Diluent Buffer: 250mM sucrose, 75mM KCl, 0.25mM spermidine, 0.25mM spermine) to 300 sperm/μl (amount for injection) and loaded into the transplantation needle. A piece of Tygon tubing was attached to a clipped tip to draw up to the dilute sperm suspension and back load the needle, the blunt end of the needle was then attached to the Tygon tube connected with the pump of the injection apparatus (Harvard Apparatus Syringe Pump Series Model "22") for the transplantation of the sperm nuclei into the unfertilized eggs. A flow of 10 nl/sec (3 sperm/sec) was used to carry out the injection by moving (right hand) the needle rapidly from egg to egg

and driving the eggs, injection dish (left hand), in front the tip of the needle to pierce the plasma membrane of each egg.

When the injected eggs reached the 4-cell to 16-cell stage, they were gently separated from unfertilized and uncleaved eggs and collected by drawing them with a wide-bore pasteur into a new petri dish filled with NAM/10 + 6% Ficoll. The cleaved embryos were analyzed at early stage or allowed to develop until tadpole stages to study the transgene expression and its function. After transplantation with swelled sperm nuclei, 20-40% of the eggs cleave, of which 50-70% carry on a normal gastrulation. From those 10-40% will be transgenic embryo.

Transgenesis protocol

250/500 ng linearized plasmidic DNA
4 µl sperm stock
SSB to 10 µl.
Incubate 5 min RT (room temperature)

Add. 10 µl Cytoplasmic egg extract
Add. 26 µl MSDB
Incubate 10 min RT

Dilute sperm stock in 500 µl MSDB
to 300 sperm/µl
Load needle

Artificial fertilization

A couple of females were primed and injected with hormones, as described above. The eggs were manually expelled from the frogs by gentle pressure on the abdomen. They were collected into a 60 mm petri dish and spread into a monolayer by stripping them into distilled water then removing the water with pasteur. A previously removed testis was teased with forceps and drawn gently across the eggs. They were left incubating for 5 min then distilled water was added. When the eggs were seen to be fertilized (rotation to bring animal hemisphere uppermost after 15/20 min), they were dejelled in 2% (w/v) cysteine and kept in NAM/10.

Graft technique

The operations, in agar-coated dishes, were performed on embryos at stage 14/20 NF in NAM/2 + 10 μg/ml trypsin to promote the detachment of the three embryonic tissue layers. Both the transgenic donor and the normal host were demembranated manually with sharp forceps. The demembranated embryos were incised using a tungsten needle in the posterior region next to the blastopore, at the level of the presumptive tail (Tucker and Slack, 1995). The neural plate was lifted using a hair-loop to provide access to the underlying notochord and somites or to be transplanted itself. From a GFP transgenic donor embryo segments of neural plate, notochord or somites were transferred with a clipped Gilson tip, into another agar-coated dishes filled with NAM/2 + trypsin inhibitor (Sigma bovine pancreas) waiting to be grafted. Then in the same region as the transgenic donor (orthotopic graft) a

normal host embryo was incised and the tissue to be replaced was removed. For the neural plate graft, this tissue was removed while for the other two kind of graft, notochord and somites, the neural plate was lifted and after removal of the tissue target and transplantation it was located back in place. After the incision and the removal of the tissue to be replaced, the host embryo was moved, by drawing it in a wide-bore pasteur, in a new agar-coated dish with NAM/10 + trypsin inhibitor and the tissue removed was replaced in the same position with the GFP-labelled transgenic graft. To immobilize the graft, it was covered by a cover-slip, previously slightly bent, over the top of the host. For the notochord and pre-somite graft the coverslip was positioned on the top of the neural plate flap that came to cover the underlying donor transgenic tissue. The hosts were allowed to heal in agar-coated dishes for 30 min in NAM/2 + trypsin inhibitor, to promote the recovery of the transplant, after which they were moved in NAM/10 + gentamicin (Sigma sulfate salt) 50 μg/ml for culture.

Graft experiments were performed also on tadpoles at stage 48 NF in order to label the dermis. Larvae transgenic for GFP were anaesthetised in 1/3000 MS222 and then segments of the tail skin were removed in NAM/10 + gentamicin 50 μg/ml and transplanted in a normal similar stage host tadpole with some tail skin peeled-off. Following transplantation the hosts were allowed to heal on a Petri cover dish coated with a wet sponge for 20 minutes and then moved again in Nam/10 + gentamicin 50 μg/ml.

LDL-diI injection

The use of diI coupled to low density lipoprotein (LDL) has been described by Levine et al. (2003). It enables the whole circulatory system to be labelled. Tadpoles at about stage 48 NF were anesthetised in 1/3000 MS222, placed on a Petri cover dish coated with a wet tissue and 4 pulse of 74 nl of 1mg/ml LDL-diI (Molecular Probes) was injected in the heart ventricular cavity using a micromanipulator, a Drummond micro-injector pump equipped with a glass micro-needle. They were examined immediately after the injection, 24 hours post-injection and following tail removal at 3 and 5 days post-amputation. The red fluorescence was observed with a Leica fluorescent stereomicroscope as described below.

Tail amputation

The tadpoles were anesthetized in MS222 (Sigma 3-aminobenzoic acid ethyl ester methanesulfonate salt) 1/3000 (w/v) in MilliQ water. For the entire duration of the operation they were kept in the anaesthetic solution (5 min). The amputation was carried out with a pairs of surgical scissors (Vannas straight small, John Weiss) at 50% of the tail length. The tadpoles were allowed to heal in tap water with aeration and subsequently were returned to the aquarium.

Detection of GFP expression

The GFP expression was observed on anaesthetized tadpoles with a Leica stereomicroscope equipped with UV lamp and GFP filters. The experiments were documented with a digital camera (diagnostic instrument RT spot camera) operating with Advanced Spot RT 3.0 software and the pictures were processed with Adobe Photoshop 7.0.

Specimen analysis

Tadpoles were killed with an overdose of MS222 (Sigma) and fixed overnight in Zamboni's fixative (40mM Na_2HPO_4, 120mM NaH_2PO_4, 2% PFA and 0.1 % saturated Picric Acid), washed in 70% ethanol overnight, dehydrated, and embedded in paraffin wax. A Leitz microtome was used to cut 6μm serial sections. Then the sections were dewaxed, re-hydrated and immunostained, then counter stained with haematoxylin (Sigma) and mounted in Aquatex (Merck). Tadpoles and embryos for whole mount immunostaning were fixed in Dent's fixation liquid (80% methanol and 20% DMSO [Sigma Dimethyl sulfoxide]) for 1 hr 30 min at 4°C, washed in methanol and stored a -20°C till the use.

Whole Mount Immunostaining

The embryos and larvae, fixed as previously described, were washed 2 times in PBSA (Oxoid Phosphate Buffered Saline Dulbecco A) and incubated in a final concentration of 10% H_2O_2 (sigma hydrogen peroxide 30% w/v solution) (w/v) in PBSA for 1 hr 30 min under the light to inactivate endogenous peroxidases and to bleach them. After the incubation they were washed 4 times in PBSA, twice in BBT (PBSA + 1% (w/v) BSA (Sigma Bovine Serum Albumin fraction V) + 0.1% (w/v) Triton X-100 (Sigma t-Octylphenoxypolyethoxyethanol)) and once in BBT + 5% (w/v) horse serum (Sigma) to block. Then the primary antibody was added a the concentration of 1/500 (w/v) in BBT + 5% horse serum and incubated overnight at 4°C on a shaker. The second day the solution with antibody was removed and the samples were washed 4 times in BBT and once in BBT + 5% horse serum. Then the secondary antibody (Sigma IgG anti mouse peroxidase conjugate) was added at the concentration of 1/1000 (w/v) and incubated over night at 4°C on shaker. The third day the solution was removed and the samples was washed once in BBT and 4 times in PBT (PBSA + 0.1% Tween 20 (Sigma polyoxyethylenesorbitanmonolaurate). Then the peroxidase substrate was added to develop the staining reaction, 500 μl of DAB (Amersham Pharmacia Cell Proliferation Kit 5-Bromo-2'-Deoxyuridine diluted in Phosphate Buffer 25 mg/ml) was added with 5 drops of substrate/intensifier (same supplier) and incubated for 5-10 min for the staining development, then was washed in PBS and H_2O to stop the staining reaction. After staining the samples were subjected to clearing process. They were dehydrated, incubated for 10min in

Methanol, then in Ethanol and finally in Murray's clearing agent (Benzyl acohol/ Benzyl Benzoate 1:2) to clear. The embryo and larvae were moved in a glass petri dish and their pictures were taken. Afterwards they were re-hydrated, incubated for 10 min in Ethanol, 10 min in Methanol then PBSA. They were fixed again in 10% (w/v) formalin (BDH Formaldehyde solution 38% w/v) in PBSA and kept at room temperature.

Whole Mount Immunostaining protocol

Day 1
Wash 2X 5min PBSA
Incubate in 10% H2O2 In PBSA for 1.30 hr
Wash 4X 5min PBSA
Wash 2X 1hr BBT
Wash 1X 1hr BBT + 5% horse serum
Add first antibody in BBT + 5% horse serum
Incubate ON at 4°C

Day 2
Wash 4X BBT
Wash 1X BBT + 5% horse serum
Add second antibody conj. In BBT + 5% horse serum
Incubate ON at 4°C

Day 3
Wash 1X 1hr BBT
Wash 4X 1hr PBT
Add reaction substrate
Block reaction H2O/PBSA
Clearing and pictures
Fix In 10% formalin in PBSA

BrdU injection

The tadpoles at stage 49 NF were anaesthetized in 1/3000 MS222, and injected with 2μl of the thymidine analogue 5-Bromo-2'-deoxyuridine (BrdU): aqueous solution of 10:1 ratio from the Cell Proliferation Kit (Amersham). The injection was performed at different times before fixation.

Immunohistochemistry

Sections were prepared as described above. Where necessary, antigen unmasking was performed by boiling the slides in distilled water for 3 minutes at full power in a microwave oven. The sections were washed three times for 5 min in PBS and then blocking of non-specific antigens on the slides was performed by incubating for 2 hr at room temperature with 2% Boehringher Blocking Buffer in PBS. The primary antibody, anti-GFP polyclonal antibody from Molecular Probes, was added at a dilution of 1:100 in 2% Boehringher Blocking Buffer in PBS overnight at 4°C. The myofibres were stained with 12/101 mAb (Kintner and Brockes, 1984) at 1:500 dilution. Neurons were detected with 2G9 mAb (Jones and Woodland, 1989) at 1:500 final dilution. The notochord was detected: in the embryos with MZ15 mAb (Craig, et al. 1987) and in the tadpoles with a commercial mAb anti-typeII collagene (ICN), both at the concentration of 1:500. The muscle satellite cells were stained with anti-Pax7 mAb (Developmental Studies Hybridoma Bank) at 1:500 dilution. Proliferating cells were labelled with a commercial antibody against PCNA (Proliferating Cell Nuclear Antigen) (Dako) at the concentration of 1:500. All the primary antibodies were incubated for the same time and under

the same condition. After primary antibody incubation, three washes in PBS per 5 min were performed, followed by the secondary antibody. Anti-rabbit (Sigma goat anti-rabbit IgG whole molecule peroxidase conjugate), was used at a dilution of 1:500 for 1 hr at room temperature for the polyclonal antibody against GFP, and for all the other primaries the anti-mouse (Sigma rabbit anti-mouse IgG whole molecule peroxidase conjugate) was used at 1:100 in 2% Boeringher Blocking Buffer for 1 hr at room temperature. After the incubation with primary and secondary antibody the binding was detected by enzymatic reaction carried on with the addition of a peroxidase substrate. First the slides were washed again for three times in PBS for 5 min and then the reaction substrate was added for the development of the positive staining. All the detection reactions were carried out using an AEC (3-Amino-9-ethylcarbazole) staining kit (Sigma). BrdU incorporation was revealed with anti 5-bromo-2'deoxyuridine mAb (Amersham "Cell Proliferation Kit"), antibody provided with nuclease for DNA unmasking. The secondary antibody was Sigma rabbit anti-mouse IgG whole molecule Alkaline Phosphatase conjugate and the staining reaction was carried out using Fast Red tablets (Sigma). Then the sections so treated were counterstained with haematoxylin (Sigma).

For simultaneous detection of GFP and Pax7, a fluorescence method was used. The primary antibodies were as specified above. For secondary antibodies were used a H+L IgG fragment goat anti rabbit FITC conjugated (Vecta Lab) for GFP detection and a H+L IgG fragment rabbit anti mouse Texas Red conjugate Vecta Lab) for Pax7 detection. The double

immunostaining was performed by first carrying out the immunoreaction against Pax7 and then that against GFP. The procedure was followed as described above, the fluorescent secondary antibodies were incubated for 3 hr at room temperature, the slides were mounted in Aquatex (Merck) and analysed with a Leica stereomicroscope equipped with UV lamp and GFP and RFP filters.

Immunohistochemistry on section protocol

Day 1
Re-hydratation sections
Antigen unmasking: 3 min boiling water
Wash 3X 5min PBS
Blocking: 2hr 2% Blocking Buffer (BB)-PBS
Add primary antibody in 2% BB-PBS
Incubate ON at 4°C

Day 2
Wash 3X 5min PBS
Add secondary antibody conj. in 2% BB-PBS
Incubate 1hr at RT
Wash 3X 5min PBS
Add reaction substrate

Double Immunofluorescence protocol

Day 1
Re-hydratation sections
Antigen unmasking: 3 min boiling water
Wash 3X 5min PBS
Blocking: 2hr 2% Blocking Buffer (BB)-PBS
Add first primary antibody in 2% BB-PBS
Incubate ON at 4°C

Day 2
Wash 3X 5min PBS
Add first secondary antibody fluorescent in 2% BB-PBS
Incubate 3hr at RT
Wash 3X 5min PBS
Blocking: 3hr 2% Blocking Buffer (BB)-PBS
Add second primary antibody in 2% BB-PBS
Incubate ON at 4°C
(Keep slides in darkness after fluorescence antibody add)

Day 3
Wash 3X 5min PBS
Add second secondary antibody fluorescent in 2% BB-PBS
Incubate 3hr at RT
Wash 3X 5min PBS
Cover the slides by Mounting media

Image capture

GFP was visualized in live tadpoles, anaesthetized as above, using a Leica FluoIII fluorescent dissecting microscope equipped with UV lamp and a GFP2 filter set. The immunofluorescence sections were analyzed with a Leica stereomicroscope equipped with UV source and GFP and RFP filters. Images were taken with a color CCD camera (diagnostic instrument RT spot camera) operating with Advanced Spot RT 3.0 software. The images were collected in TIFF format (Tagged-Image File Format) then processed with Adobe photoshop 7.0.

III-Description of *Xenopus* tail regeneration

Morphological analysis of the regenerating tail

In order to understand completely the mechanisms occurring during tail regeneration a clear understanding is needed of the morphology of the regenerating tail. Several regenerating *Xenopus laevis* tadpoles were sacrificed, processed and then sectioned, as described in materials and methods, two, three and five days after tail removal in order to analyze their organization. The cases shown in Fig.III1 are regenerating *Xenopus* larvae stained with haematoxylin and with 12/101 antibody in order to highlight the muscle fibres and give a more clear picture of the tissue composition of the regenerate. It is very clear that there is a specific compartmentalisation of at least the spinal cord and the notochord in the regenerate. So I will refer to the regenerating tail tissues as a "regeneration bud" (Gargioli and Slack, 2004) rather than as a blastema; the term "blastema" will be reserved for the undifferentiated mesenchymal part of the regeneration bud. The importance of the wound healing epithelium as stimulating structure during the regenerative processes has already been mentioned. Recent publications highlight the different structure of a wound healing epithelium, a wound epithelium and their relationship to regeneration capability. Beck et al. (2003) demonstrated that *Xenopus* tadpoles present stage dependent regenerating and non regenerating capability in the tail, as well as limbs (Dent, 1962;

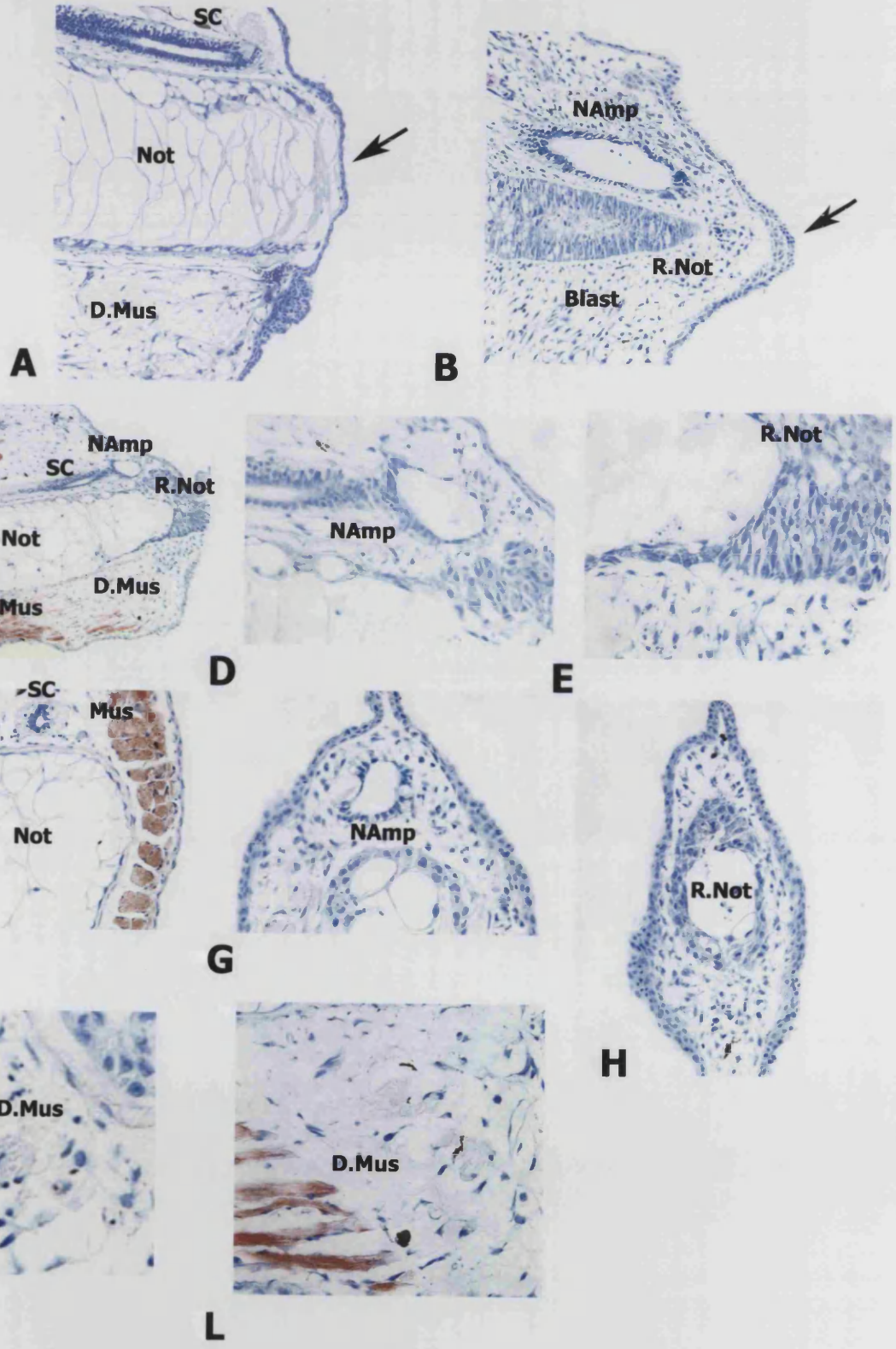
Muneoka et al, 1986; Overton, 1963). In the limbs there is a gradual loss of the regenerating capability with the progress of development. Regeneration of the limbs becomes lost at later stages, coincident with the onset of ossification of the limb skeleton (Wolfe et al., 2000). For the tail Beck et al. (2003) demonstrated that there is a window, a refractory period, when the tadpoles lose the capacity to regenerate the tail (stages 46/47 NF). Although the *Xenopus* tadpoles can regenerate their tails up to metamorphosis, the embryonic tail bud can not completely regenerate (Tucker and Slack, 1995) and even a removal of a small part of the tail bud causes a defect in the final tail. Thus given this discordance of behaviour between embryos and tadpoles they investigated the tail regeneration of different tadpoles stage: stages 42, 46/47 and 48 NF, showing that exactly at stage 46/47 NF the *Xenopus* tadpoles lose this capability present at stage 42 NF and also display from stage 48 NF up to metamorphosis. Beck showed that the tail regenerating capability is associated with the re-expression of specific genes playing a pivotal role during the tail development such as BMP and *Notch*. Moreover she presented some morphological analysis regarding the epithelium covering the amputated surface. Following tail removal, after 6-12 hours, a wound epithelium forms rapidly covering the amputated surface over the cut. Within 24 hours undifferentiated cells appear immediately beneath this epithelium becoming several cells thick. In 48 hours a morphological cone-shaped bud forms, which is actively proliferating and rapidly expanding, giving rise to the regenerating tail. In non regenerating tadpoles (stages 46/47 NF) a completely different situation is presented. The epithelium covers the wound

very slowly and by 24 hours post-amputation, a skin-like wound epithelium has formed over the damaged surface (Beck et al., 2003). Thus the structure of the wound healing epithelium is correlated with the regeneration processes as mentioned by Neufeld (1989). In fact the results reported in his research show that a blastema, characteristic of amphibian epimorphic regeneration, also exists in nonregenerating mice. Several such features were found. A small area of wound bed was covered by wound epithelium which initially lacked an underlying basement membrane, as in regenerating limbs. Blastemalike growth was revealed in the subdermal layer surrounding periosteal chondrogenic cells. Mesenchymelike cells were seen among the fibroblasts and leucocytes within the proliferating tissues. However the dermis persisted as apparent intact obstruction to growth bud (blastema) formation, impairing the complete development of the regenerating "machinery". In Fig.III1 A,B are shown the *Xenopus* tadpole wound healing epithelium at two and five days post amputation. It is initially one cell thick and later a few cells thick. The cone-shaped bud which forms relatively quickly after tail removal was thought to be a blastema formed by a mass of actively proliferating, undifferentiated, pluripotent cells giving rise to all tissue of the new re-forming tail during the *Xenopus* tail regeneration process. By analysing the histological section presented in Fig.III1, it appears obvious that this is incorrect. In fact the specimens, at three days after tail amputation, showed that the spinal cord closes off and forms a terminal neural ampulla (Fig.III1C,D) This was previously described by Stefanelli (1951) as the "ampulla apicalis" continuous with stump cord, during tail regeneration of

amphibians and reptiles. The notochord terminates with a bullet-shaped mass of cells in continuity with the notochord epithelium sheath of the more proximal tissue (Fig.III1E). Thus as mentioned above those two tissues appear to keep their morphological architecture as specific closed compartments. The muscles, especially the myofibres in the vicinity of the amputation surface (1mm) seem to degenerate. In longitudinal and transversal sections is clearly present a large amount of cellular debris and acellular protein masses accumulated over the first three days (Fig.III1I,L). Over the same period, a mass of undifferentiated cells migrate and colonise a ring region around the neural ampulla and the notochord tip, particularly well visualized in Fig.III1H. I will refer to this undifferentiated regionalised mass of cells as a "blastema" and to the whole regenerating region as a "regeneration bud" (Gargioli and Slack, 2004).

Fig.III1 Longitudinal and transversal sections of regenerating tail immunostained with 12/101 mAb and counterstained with Haematoxylin at different stages: longitudinal at two days and five days **(A, B)**; three days longitudinal **(C-E, L)** and transversal **(F-I)**. **(A)** Two days regenerating tail section showing a very thin wound healing epithelium (black arrow), spinal cord (SC), notochord (Not) and degenerating muscle (D.mus). **(B)** Regeneration bud at five days after tail removal presenting a thickened wound healing epithelium (black arrow) covering the blastema, also called "apical cap" (Gardiner et al., 1986), neural ampulla (NAmp), regenerating notochord (R.Not) and the undifferentiated cells of the blastema (Blast). **(C)** Low-power magnification showing neural ampulla (NAmp), regenerating notochord (R.Not) and blastema (i.e. visibly undifferentiated cells) formation close to the cut surface. **(D)** Enlarged view showing details of the NAmp in A. **(E)** Enlarged view showing the details of the R.Not in A. **(F)** Stump transversal section, proximal to amputation surface, showing notochord (Not), spinal cord (SC) and muscle (Mus). **(G)** Detail of the enlargement of the ependymal canal in the NAmp. **(H)** Transverse section of distal to amputation level showing R.Not and the surrounding undifferentiated blastema cells. **(I,L)** Degenerating muscle (D.Mus) near the amputation level. Scale bars: 100 μm.

Fig.III1

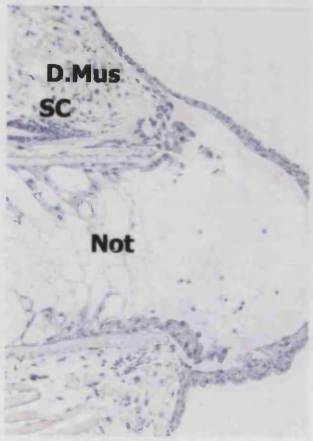

SC
Not
D.Mus
A
NAmp
R.Not
Blast
B
NAmp
SC
R.Not
Not
D.Mus
Mus
C
NAmp
D
R.Not
E
SC
Mus
Not
F
NAmp
G
R.Not
H
D.Mus
I
D.Mus
L

Identification of dividing cell populations

BrdU incorporation

To analyze in detail the proliferating cell populations and those actively taking part in the regeneration process, the thymidine analogue 5-Bromo-2'-deoxyuridine (BrdU) was injected intraperitoneally in tadpoles at stage 49 NF as described in materials and methods. Two types of labelling were carried out: a short time injection (twenty-four hours before fixation) and a long time injection (one day before tail removal). The tadpoles so treated were sacrificed at two and five days after the tail removal in order to label the whole set of cell populations taking part to the formation of the regeneration bud. By immunostaining with a monoclonal antibody against BrdU, incorporation was detected on sections. The two different labelling times were chosen in order to highlight differences among the dividing population in the pre-amputation tail and during tail regeneration. In Fig.III2 are presented sections from tadpoles treated for the immunodetection of BrdU at two and five days after tail removal, and short and long time exposure. There are not significant difference between the two series of experiments regarding the time of exposure to BrdU, except for the major dilution of the BrdU in the long treated larvae. The proliferating cells take up the BrdU, but the active cellular division leads to dilution of the BrdU incorporated at time zero then to further dilution depending on the number of cell cycles. Thus the difference in numbers and intensity of the BrdU positive cell signal is due to the active cell division of the tissues in the regeneration bud. No difference in terms of different cell populations labelled was seen in the two type of treatment.

Fig.III2



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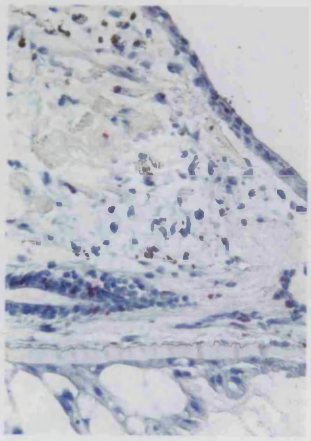

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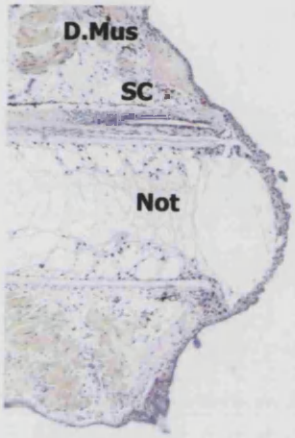
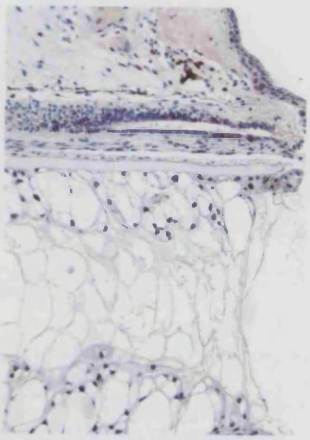
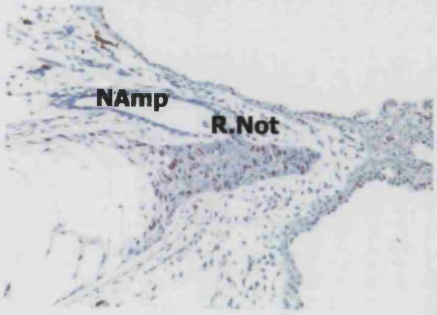


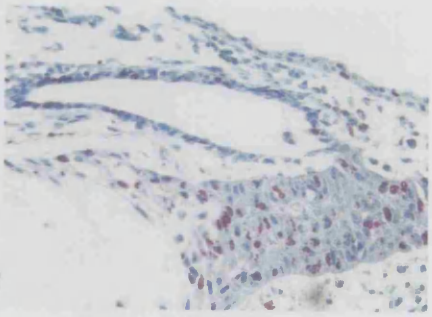
C



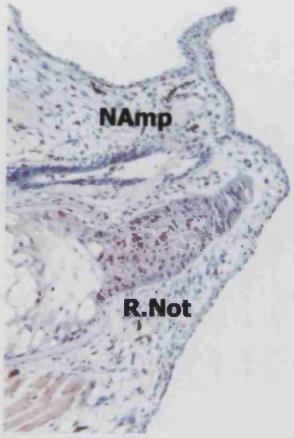
D



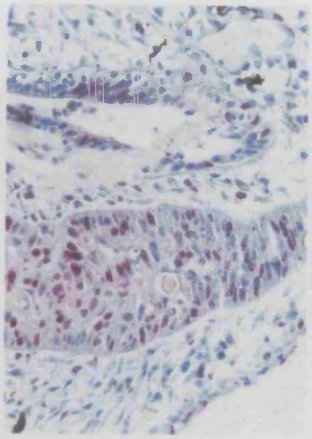
E



F



G

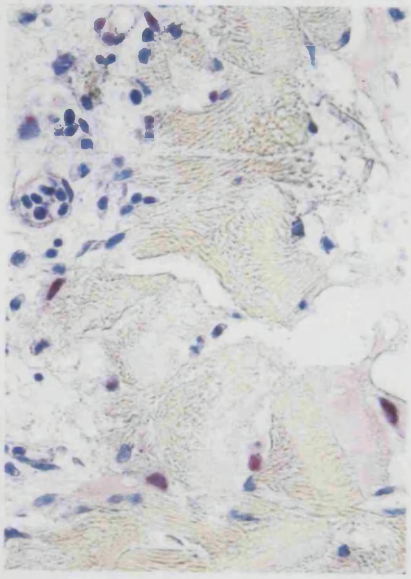


H

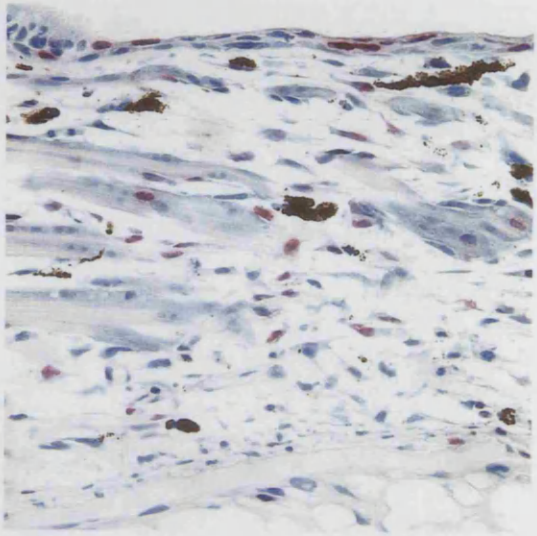
Fig.III2 Immunohistochemistry on longitudinal sections of regenerating tail for long and short BrdU treated Xenopus tadpoles at different regeneration stages: two days after amputation long **(A,B)** and short BrdU exposure **(C,D)**; five days after tail removal long **(E,F)** and short BrdU treatment **(G,H)**. **(A,C)** Low power magnification showing BrdU incorporation in the regeneration bud: in the spinal cord (SC) and notochord (Not); and presenting degenerating muscle (D.Mus). **(B,D)** Enlarged view of respectively (A) and (C), showing details of SC and the BrdU positive cells in the undifferentiated blastema. **(E,G)** Low power magnification showing BrdU incorporation in the reforming structure of the regeneration bud: neural ampulla (N.Amp) and regenerating notochord (R.Not). **(F,H)** Enlarged view of respectively (E) and (G), presenting in more details the BrdU immunodetection in N.Amp and R.Not.

By analysing in detail the single tissues labelled by BrdU detection the undisturbed architecture of the spinal cord and notochord is clear again and contrasts with the massive degeneration of the muscle fibres close to the cut level (1mm). At two days after tail removal (Fig.III1A-D) there is not much regenerated tissue, nevertheless the incorporation of the BrdU in the stump tissue close to the amputation surface is already visible. In the ependymal cell layer of the spinal cord there is a high rate of cell proliferation, and in the notochord, and in the undifferentiated cells surrounding the notochord. As previously described the number of positive cells is much higher for samples derived from the short time exposure, due to the high rate of cell proliferation during the regenerating process,. At five days post amputation the BrdU incorporation increases remarkably in the regenerating notochord and in the ependymal canal of the spinal cord (Fig.III1E-H). The epidermis as well presents high BrdU incorporation at two and five days, due to the high rate of the natural turnover and cell renewal in this tissue type. At two days post amputation the massive muscle degeneration is apparent (Fig.III2B,C).

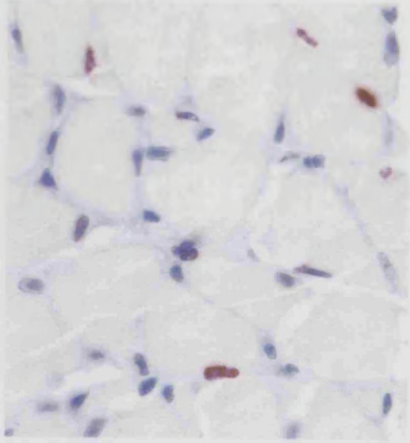
Fig.III3



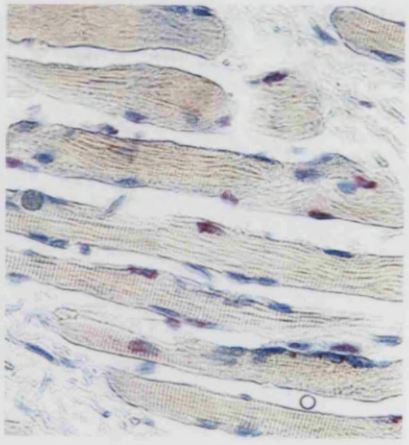
A



B



C

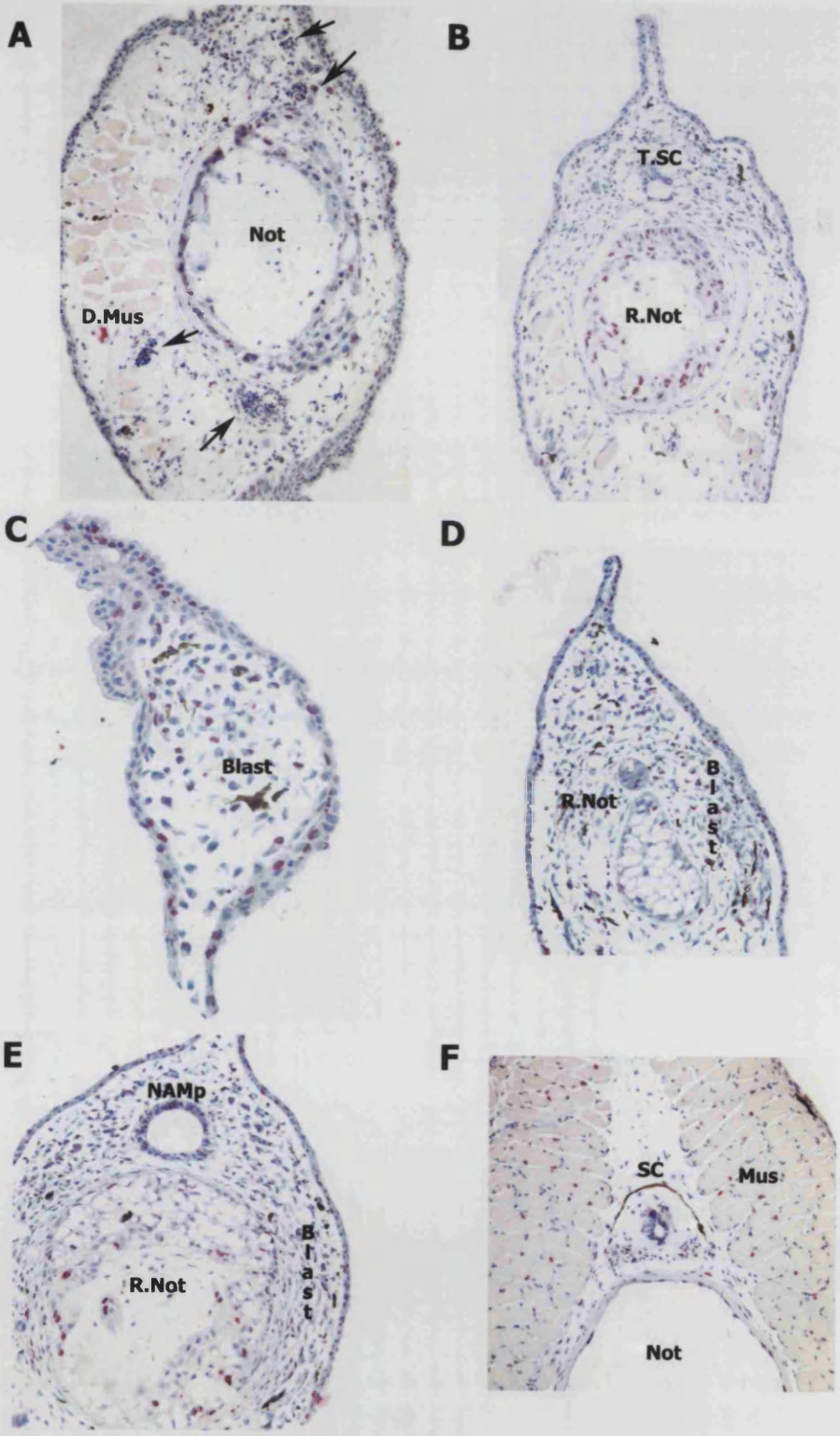


D

Fig.III3 Brdu incorporation in *Xenopus* tadpoles regenerating **(A,B)** and non regenerating muscle **(C,D)** shown by immunostaining and counterstained with Haematoxylin; BrdU positive cells in red. **(A)** Transverse section at two days after tail removal, presenting massive muscle degeneration close to the amputation surface and few BrdU positive cells in the undamaged muscle fibres. **(B)** Longitudinal section showing positive red cells in the undamaged myofibres and some migrating to the blastema. **(C)** Transverse section and **(D)** longitudinal section of mature myofibres showing BrdU incorporation as well.

The muscle fibres close to the cut surface seem to degenerate and the environment appears to contain widespread acellular protein and cellular remains. In Fig.III3 are shown muscles in transverse and longitudinal sections, at two and five days after tail removal. The degenerating muscles are clearly visible with just few cells on the peripheral edge of the undamaged muscle fibres showing BrdU incorporation. The non-regenerating muscles in transverse section shown the same behaviour, indicating cell proliferation apparently in the differentiated myofibres, but again in the same position: at the edge of the fibres in flat nuclei positioned peripherally to the muscle fibre area (Fig.III3C). In the vicinity of the amputated surface at five days the muscle fibres appear undamaged and elongated like in the normal, non regenerating myofibres. The degree of BrdU incorporation is higher in the regenerating muscles and some of the positive cells are outside but in the vicinity of the muscle fibres, in tight contact, giving the impression of migrating from the undamaged myofibres (Fig.IIIB). In longitudinally sectioned non-regenerating differentiated muscles the active cell proliferation is also visible, as shown by the BrdU incorporation in mature fibres,

Fig.III4


A
Not
D.Mus
B
T.SC
R.Not
C
Blast
D
R.Not
Blast
E
NAMp
R.Not
Blast
F
SC
Mus
Not

nevertheless the cell type actively synthesizing DNA is less clear using this plane of section (Fig.III3B,D).

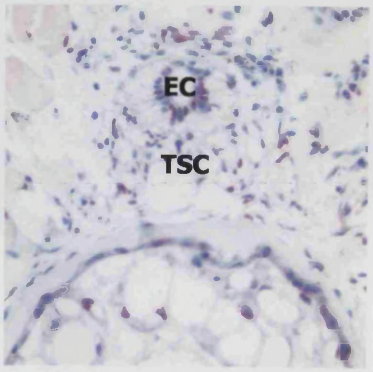
Fig.III4 BrdU immunodetection on series of transversal sections running proximal to distal relative to the amputation surface at two **(A,B)** and five days **(C-F)** after tail amputation (BrdU in red). **(A)** Proximal section showing red BrdU positive cells in the notochord (Not), presenting also degenerating muscle (D.Mus) and several inflammatory foci (black arrows). **(B)** Distal section presenting the truncated spinal cord (T.SC) with the enlargement of the epandymal canal and BrdU positive cells in the regenerating notochord (R.Not). **(C)** Highly proximal section showing many Brdu positive cells in the undifferentiated blastema (Blast). **(D)** Proximal sections showing Blast and R.Not both positive for BrdU incorporation. **(E)** Less proximal section presenting neural ampulla (N.Amp), R.Not and Blast BrdU positive. **(F)** Highly distal section showing mature tissue: SC, Not and Mus with a certain degree of BrdU incorporation.

Fig III4 shows a series of transverse sections at two and five days post amputation: running proximal to distal relative to the amputation surface. The difference in quantity of tissue present at the two time points is obvious, as is the integrity of the tissue and the rate of cell division. In proximal sections at two days post amputation (Fig.III4A,B), is present a high number of inflammatory cells probably migrating to the vicinity of the damaged surface; and also a quantity of extracellular proteins and matrix. BrdU incorporation level is not that high due to the extreme damage and the lack of regenerating tissue. Also evident is the degree of muscle degeneration and disorganization compared with the more distal section (Fig.III4A). More distally the active dividing wide open ependymal canal of the spinal cord and the proliferating stump of the notochord is visible, (Fig.III4B). At five days the organization of the tissue regenerate is more clearly defined, distally the regeneration bud is formed just of undifferentiated blastemal cells rich in BrdU positive nuclei

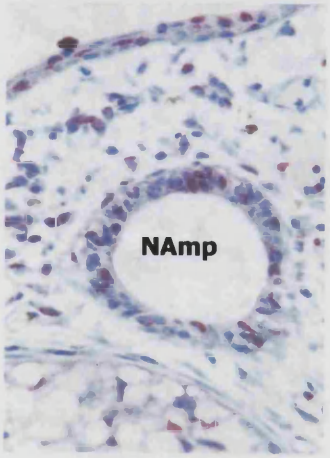
(Fig.III4C). By going proximally, it is possible to see first the regenerating notochord tip and then the neural ampulla (Fig.III4C-E), both incorporating BrdU. In Fig.III4F a proximal section, far away from the amputated surface, shows the normal tail tissue organization, presenting as well a certain degree of BrdU incorporation into nuclei associated with the muscle fibres. More details of the spinal cord, the neural ampulla and the notochord show that it is possible to locate a proliferating cellular subpopulation within each tissue. In fact examination of the neural ampulla and its base (Fig.III5A) suggests that the high proliferation in the base of the neural ampulla pushes the ependymal layer to form this swollen structure. The neural ampulla cells are BrdU positive, nevertheless the incorporation is much less than the base of the ampullar formation. In fact the cell counting experiments reported in table III1 show that the labelling index in the base of the neural ampula is about two fold the labelling index in the main body of the neural ampulla. Furthermore the BrdU signal in the neural ampulla resembles the normal spinal cord, with actively proliferating cells at the level of the ependymal canal, exactly as in the ependymal cell layer covering the cord canal (Fig.III5C,D). Regarding the notochord, it also seems that the cell subpopulation of the outer notochord is of the main source of cells for notochord regeneration as it proliferate with a higher level than the rest of the notochord, (Fig.III5D,E).

Fig.III5

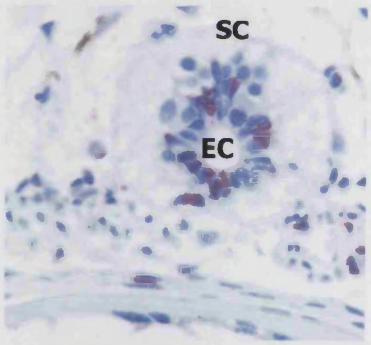
A



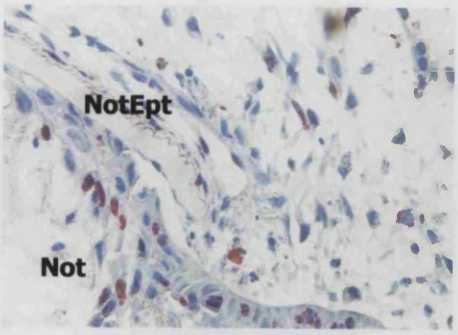
B



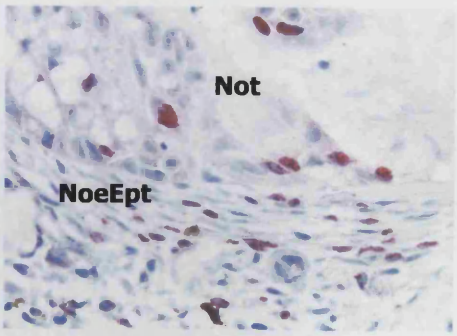
C



D



E



F

Table III1. BrdU labelling index in the regenerating SC: Base of N.Amp and N.Amp (1000 nuclei were count)

	N.Amp	Bas. N.Amp
BrdU-positive nuclei (%)	16.6	47.3
PCNA-positive Nuclei (%)	17	52

Fig.III5 Immunohistochemistry against BrdU (red signal) in longitudinal **(A,E)** and transversal sections **(B,C,D,F)** of regenerating tail at different stages. **(A)** Large view of regenerating notochord (R.Not) and base of the neural ampulla (Bas N.Amp) being positive for BrdU, at five days post amputation. **(B)** Section of a proximal amputation surface level at two days post amputation showing the truncated spinal cord (T.SC) and in particular the BrdU positive ependymal canal (EC) of the SC. **(C)** Large view of a five days N.Amp BrdU positive. **(D)** Large view of a distal amputation surface level SC presenting several BrdU positive cells in the EC. **(E,F)** Particular of the regeneration bud at five days after tail removal showing the R.Not positive and the notochord epithelium (NotEpt) highly positive for the BrdU incorporation.

PCNA positive proliferating cells

In order to confirm the results on the morphology and proliferative activity of the regeneration bud, immunostaining against the Proliferating Cell Nuclear Antigen (PCNA) was performed. A commercial antibody, as described in materials and methods, were used on regenerating tail sections at two and five days post amputation as shown in Fig.III6. At two days after tail removal the small amount of regenerating new tissues is visible around the damaged notochord (Fig.III6A). The spinal cord presents a wide open ependymal canal close to the amputation surface and, as seen previously, the muscle fibres in the vicinity of the cut area are completely degenerating and associated with large amounts of cell debris and acellular material (Fig.III6B). The PCNA-positive cells appear numerous and intensely immunostained, particularly in

the ependymal cell layer of the spinal cord, in the notochord, close to notochord sheath and in the epidermis (Fig.III6C). The sections resulting from five days post- amputation larval tails show the complete structure of the tail regeneration bud: the unperturbed architecture of the regenerating notochord, with a bullet-shaped tip; the regenerating spinal cord ending with the neural ampulla and the undifferentiated blastemal cells surrounding both those structures (Fig.III6D). By observing in detail the different structures it appears that localization of the cell proliferation is the same as seen with the use of BrdU (Fig.III2.). The regenerating notochord, beneath the neural ampulla, is still immature, non-vacuolated, and is completely positive for PCNA. The regenerating spinal cord ends with the neural ampulla, presenting a moderate proportion of PCNA-positive cells, whereas at the base of the neural ampulla, corresponding to the caudal end of the ependymal cell layer of the spinal cord, it is shown high proportion of positive cells (Fig.III6E,F). This indicates the high proliferation rate in the ependymal layer cells of the neural tube leading to the neural ampulla. At five days after amputation the muscle appears with a completely different organisation than after two days (Fig.III6G). The fibres present the characteristic elongation and some regenerating new myofibres show intense PCNA positive reaction. As was seen for BrdU incorporation, the normal myofibres in the rostral region of the tail also show many PCNA positive nuclei, although these are flat in shape and peripheral to the myofibres (Fig.III6H).

Fig.III6

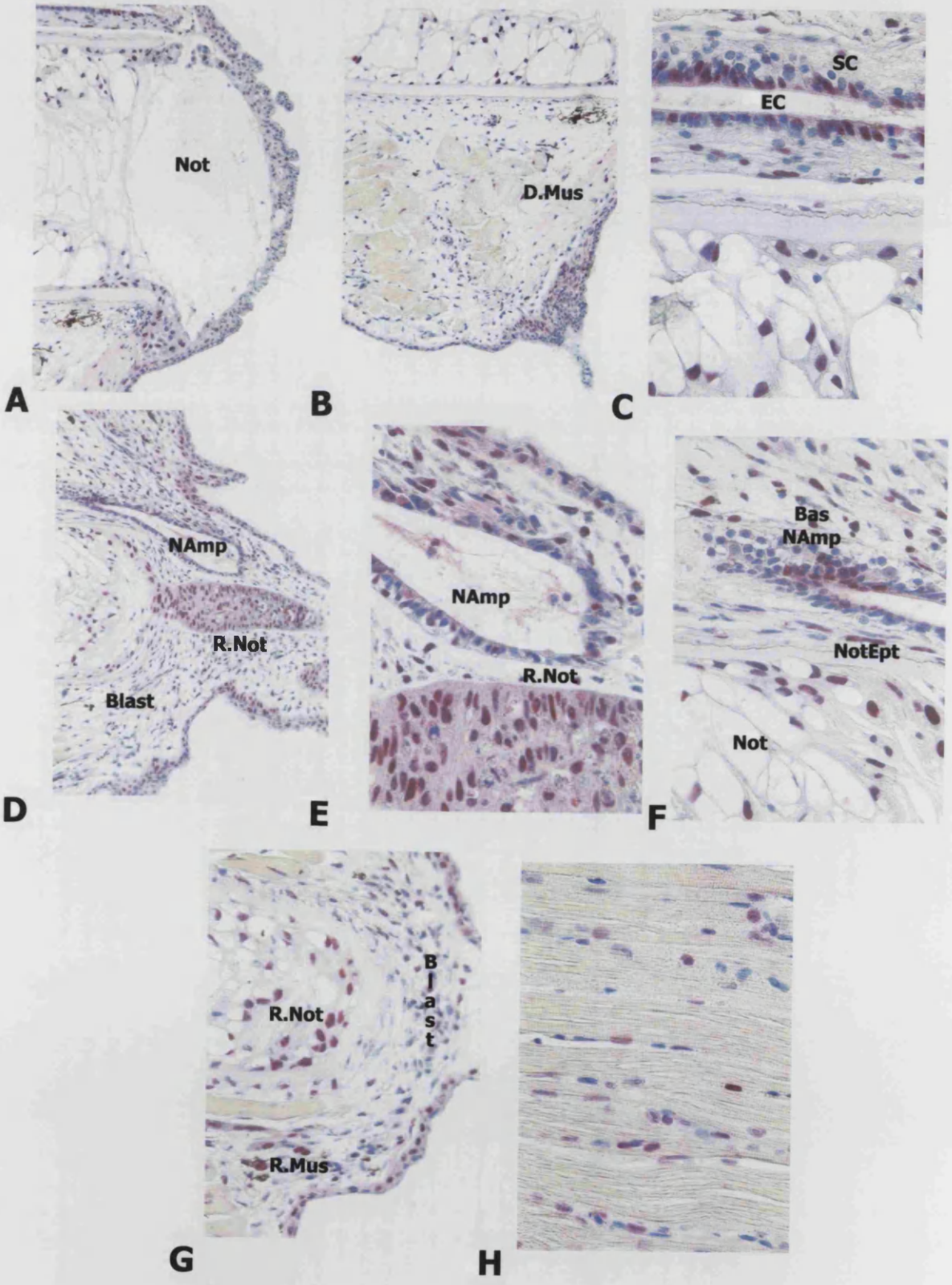

Not
A
D.Mus
B
SC
EC
C
NAmp
R.Not
Blast
D
NAmp
R.Not
E
Bas
NAmp
NotEpt
Not
F
R.Not
Blast
R.Mus
G
H

Fig.III6 Immunodetection of PCNA counterstained with Haematoxylin on longitudinal sections of regenerating *Xenopus* tadpoles tail at two **(A-C)** and five days **(D-H)** after tail amputation. **(A)** Low power magnification showing the notochord (Not) and the surrounding tissues being PCNA positive (red). **(B)** Enlarged view of (A) showing degenerating muscle (D.Mus) with few PCNA positive cells. **(C)** Enlarged view of (A) showing the spinal cord (SC), in particular the ependymal canal (EC) highly positive for PCNA. **(D)** Complete view of the positive PCNA cells in the regeneration bud tissues: neural ampulla (N.Amp), regenerating notochord (R.Not) and undifferentiated blastema (Blast). **(E)** Enlarged view of N.Amp and the R.Not in (D) showing high PCNA positive cell rate. **(F)** Enlarged view of (D) presenting in particular the high PCNA positive cell density in the base of the neural amoulla (Bas N.Amp) and in the notochord epithelium (NotEpt). **(G)** Lateral view of the regeneration bud showing PCNA positive cells in the R.Not, in the Blast and in the regenerating muscle (R.Mus). **(H)** Mature myofibers present PCNA positive red cells as well.

In proximal-distal serial sections at two and five days it is possible to compare the difference of tissue amount and tissue organization at early and later stages of the tail regeneration process (Fig.III7).

The distal sections, in the vicinity of the amputated surface, show the regenerating notochord tissue close to the notochord sheath. Underneath the re-growing notochord a mass of undifferentiated blastemal cells appear, above those structure is visible the enlarged ependymal canal which terminates the spinal cord. In the muscle flanking both those structures it is possible to recognise the signs of a massive inflammation by the amount of small macrophages and leukocytes and the muscle degeneration by the empty space filled with extracellular proteins and matrix (Fig.III7A). In the sections proceeding to the more proximal area of the regenerating tail, the different organisation and morphology of the normal muscles is seen, lacking the inflammatory cells and empty gaps among the muscle fibres (Fig.III7B). Also the other tail core structures, spinal cord and notochord present a

different morphology: the spinal cord has a normal ependymal canal and the notochord has a normal organisation wiht big and tonic vacuoles. In serial sections for five days amputated larvae, the portion of tissues positive for PCNA is much higher (Fig.III7C-E). Distally the regenerating notochord stands out from the regeneration bud for the high positive signal for PCNA, surrounded by undifferentiated blastema cells. Continuing in proximal direction the next clearly recognisable structures are the neural ampulla on the top of the notochord and some differentiated myofibres flanking the notochord (Fig.III7C). No inflammatory cells are visible at this stage of the regeneration process. In a proximal section, faraway from the cut site, the complete normal organisation of the tail is shown, with PCNA positive cells in the ependymal layer of the spinal cord, in the outer notochord and in the notochord sheath and in the muscles, as seen in the BrdU immunostaining (Fig.III7E). In Fig.III8 are shown the details of the regenerating tissues in particular showing the specific localisation of the high PCNA positive zone. The neural ampulla shows just in few PCNA positive cells (Fig.III8A,B). The neural ampulla shown in this panel is at early stage of formation presenting a very thin monocellular layer with two positive cells. This structure will become thicker following colonisation of the cells from the base of the ampulla (Fig.III6F). the notochord shows the highest signal for cell proliferation in the outer notochord (Fig.III8 C,D). There are few PCNA positive nuclei inside the notochord structure. The muscles (Fig.III8E,F), show the massive degeneration of the myofibres at early stages of the tail regeneration process with numerous inflammatory cells.

Fig.III7

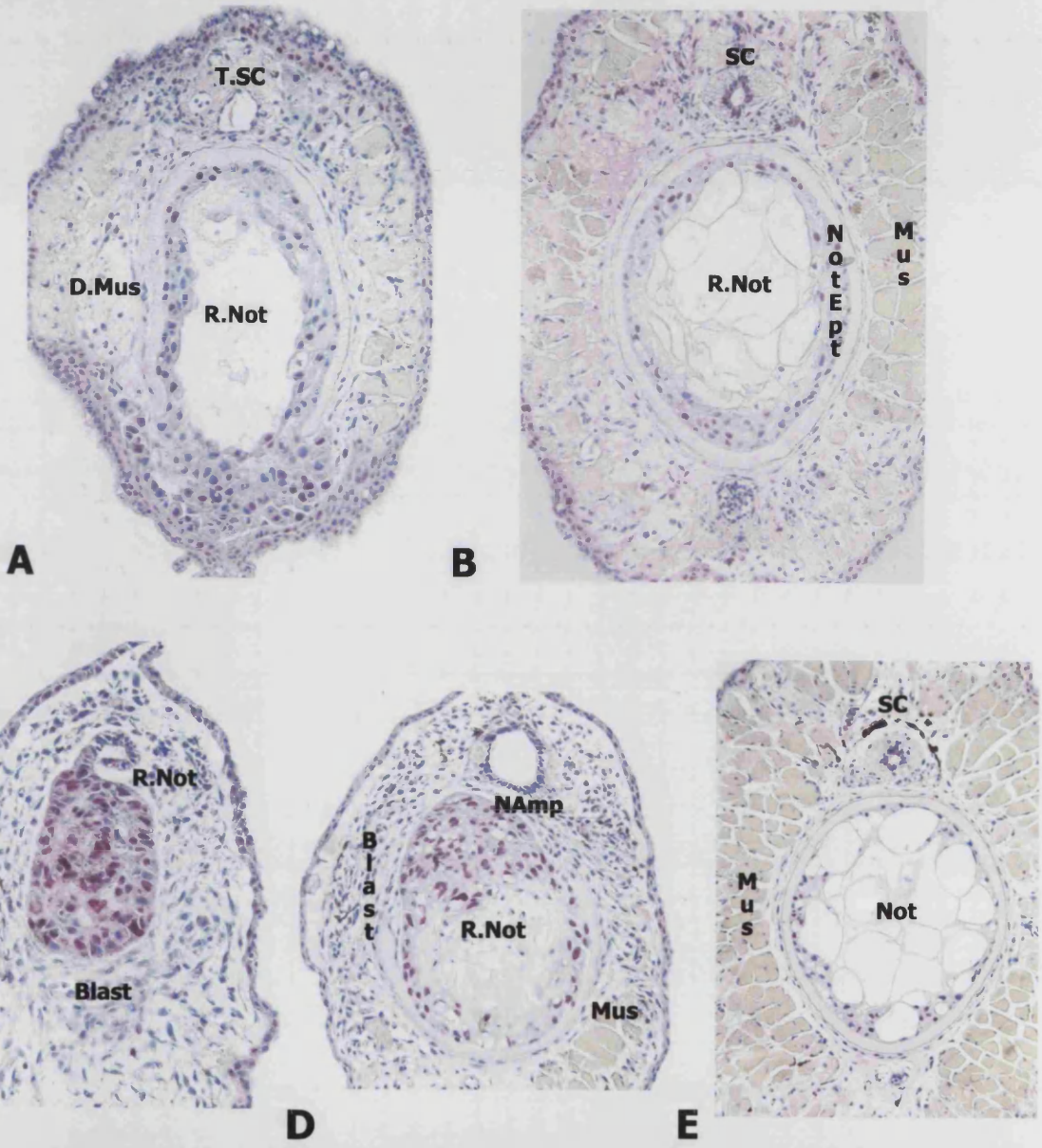
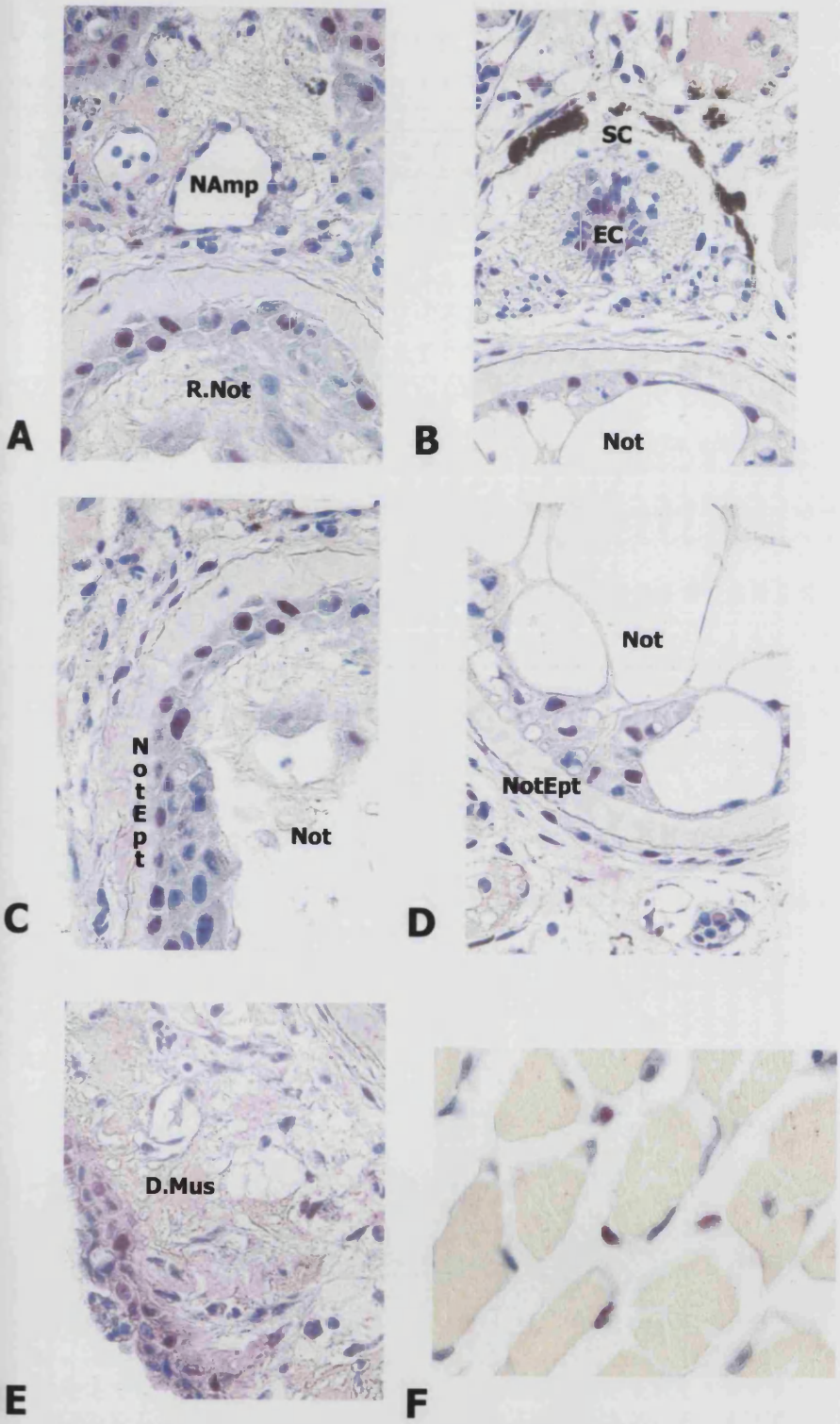

T.SC
D.Mus
R.Not
A
SC
R.Not
NotEpt
Mus
B
R.Not
Blast
C
NAmp
Blast
R.Not
Mus
D
SC
Mus
Not
E

Fig.III7 PCNA immunostaining on series of transversal sections running proximal to distal relative to the amputation surface at two **(A,B)** and five days **(C-E)** after tail amputation (PCNA in red). **(A)** Proximal section showing red PCNA positive cells in the regenerating notochord (R.Not), presenting also degenerating muscle (D.Mus) and a truncated spinal cord (T.SC) **(B)** Distal section showing muscle (Mus), SC positive in the ependymal canal for PCNA as R.Not and notchord epithelium (NotEpt). **(C)** Proximal section showing several PCNA positive cells in the undifferentiated blastema (Blast) and in the R.Not. **(E)** Less proximal section presenting neural ampulla (N.Amp), R.Not, Mus and Blast being PCNA positive. **(F)** Highly distal section showing mature tissue: SC, Not and Mus with several PCNA red positive cells.

Transverse sections of normal muscles show the normal organisation with flat PCNA positive, proliferating, nuclei located at the edge of the muscle fibres, exactly as shown for the BrdU positive muscle cells.

Fig.III8
Immunohistochemistry on transversal sections of *Xenopus* regenerating tadpoles against PCNA (red). **(A)** Section of proximal amputation level showing new formed neural ampulla (N.Amp) and regenerating notochord (R.Not) positive for PCNA.**(B)** Distal level section presenting the spinal cord (SC) with the PCNA positive ependymal canal (EC) and the Not. **(C,D)** Respectively sections of proximal and distal amputation level showing PCNA expression in the notochord epithelium (NotEpt) and in the Not. **(E)** Section at proximal amputation level showing degenerating muscle (D.Mus) being negative for PCNA. **(F)** Mature muscle fibres presenting PCNA positive red cells.

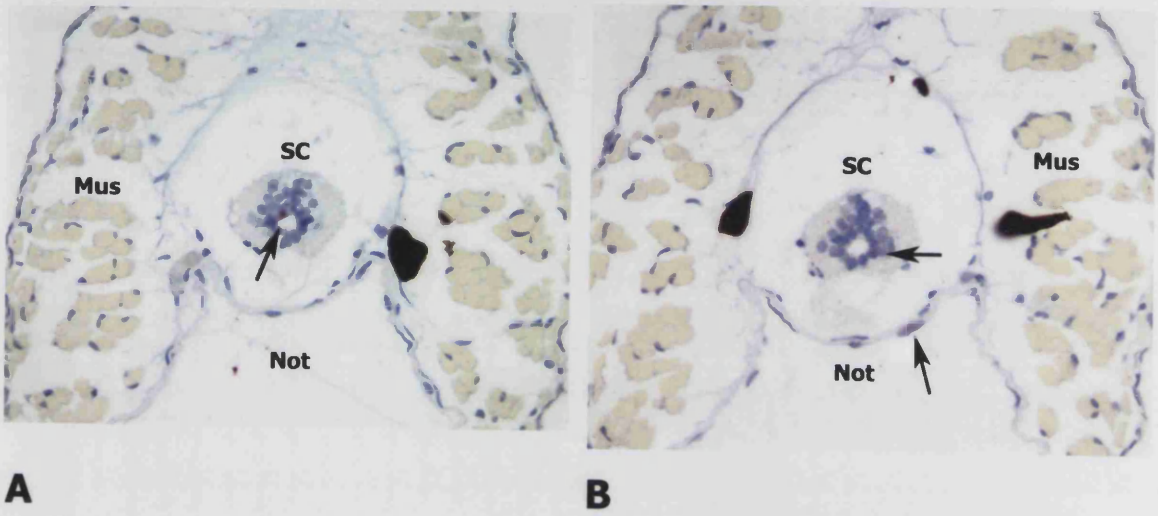
Fig.III8



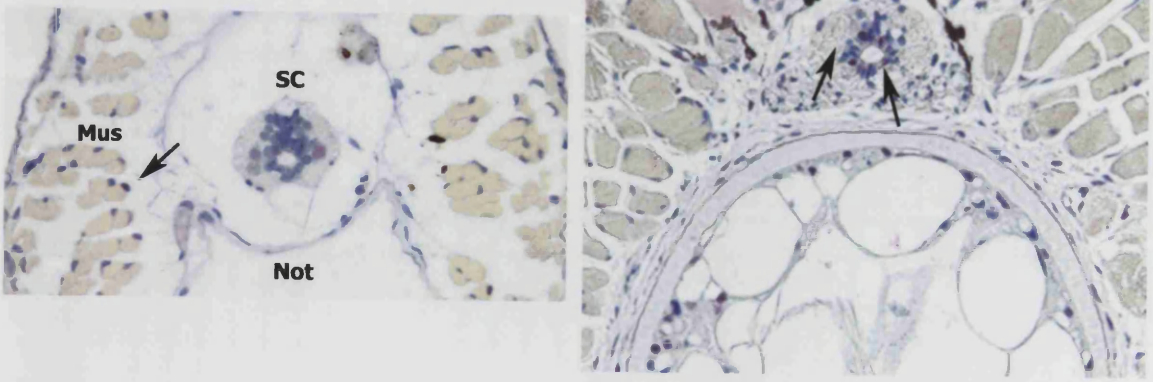
Refractory Period

Previously I have already mentioned that the embryonic tail is normally able to regenerate all the tail core tissue (neural tube, notochord and muscles) whereas this capability is consistently lost between stage 45 and 47 NF. To examine the proliferative status during the refractory period, transverse sections from amputated tadpoles were examined by immunostaining against PCNA. In Fig.III9A-C are shown three different transverse section from non-regenerating larvae at five days post-amputation. Consistent with the gross morphological description given by Beck (2003) , sections next to the amputation surface do not show any presence of regenerating tissue or morphological changes in the tissue organization (Fig.III9A-C). The tissue damage caused by the tail cut has been recovered by the skin, and the tail core tissues heal the injury without forming a regenerate. In proximal sections the tail structure is normal but there is an almost complete absence of PCNA positive signal. Normal tail sections at stage 48 NF were used as positive control presenting the normal PCNA positive cells in the ependymal canal of the spinal cord, in the notochord and in the edge of the muscle (Fig.III9D). In the stage 46/47 NF tail sections (Fig.III9A-C) is clearly visible the incomplete organisation of the muscle fibres presenting numerous gaps, the small area of mature tissue of the notochord and the lack of a complete grey matter in the spinal cord compared with more mature structural organization of the stage 48 NF (Fig.III9D). PCNA reactivity is visible in few cells of the spinal cord, notochord and muscle, serving as a positive control for the staining. By counting the red PCNA-positive nuclei in different tadpole tail

Fig.III9



A B



C D

sections of the refractory period and of the regenerating stages is revealed a remarkable difference of PCNA-positive signal, with a much decreased active cell proliferation in the tail tissue of non-regenerating larvae (Table III2). Therefore the loss of the regenerative capability of the tail for *Xenopus* tadpoles during the refractory period is probably due to the fact that in this developmental window occurs also a considerable decrease of cell proliferation.

Table III2. Ratio of PCNA-positive nuclei (1000 nuclei were counted)

	Stage 46-47 NF			Stage 48 NF		
	SC	**Not**	**Mus**	**SC**	**Not**	**Mus**
PCNA positive nuclei (%)	3,6	1,8	0,9	45,7	25,3	20

Fig.III9 Transverse sections of non regenerating **(A-C)** and regenerating **(D)** *Xenopus* tadpoles immunostained for PCNA positive cells (red). **(A)** Section showing the tail core tissue: spinal cord (SC), notochord (Not) and muscles (Mus); with just one PCNA positive cells in the SC (black arrows). **(B)** Section presenting the tail tissues with few positive PCNA cells in the Not and SC (black arrows). **(C)** Section showing one positive cells for PCNA in the Mus (black arrow). **(D)** Control section showing high PCNA positive cells in the SC (black arrows) in the normal regenerating tadpoles tail.

Conclusion

Under the evidence described in this section it is possible to describe the tail regeneration process in its morphological aspect. This allows some tentative conclusions about cell lineage, which will be amplified by the tissue labelling studies presented in the later chapters.

Spinal cord

The regenerating spinal cord seems to take origin from the stump spinal cord. The specific compartmentalisation of this tissue, preserving its architecture, suggests the isolation of the cord during the tail regenerating process. Apivotal role during the tail regeneration is played by the swelling of the ependymal canal forming the neural ampulla. This structure is at the base of the regenerating spinal cord, as described by Stefanelli (1951) for spinal cord regeneration in urodele amphibians and reptiles. This ampulla resulting by the proliferation of the ependymal cell layer, covering the spinal canal, starts the regenerative process for the spinal cord.

Notochord

The regenerating notocord, as the spinal cord, is continuous with the stump notochord. Early in the tail regeneration process the notochord terminates with a bullet-shaped mass of cells in continuity with the outer notochord of the more proximal tissue. This regenerating notochord shows characteristics different from the mature one. Normally the notochord is a completely vacuolated structure, wheras the regenerate shows a plain mass of cells forming a compact structure, vacuolating later during the regeneration process.

Muscles

For the muscles the process is completely different and the only clear evidence arising from the histology is the massive degeneration of the myofibres in the vicinity of the cut surface, with the presence of a large amounts of cellular debris and acellular protein masses. In spite of this degenerated muscles, some undifferentiated cells are visible close to the damaged surface, located in a ring around the notochord and spinal cord: the blastemal cells.

BrdU and PCNA

The experiments conducted with BrdU injection and PCNA immunodetection revealed the high proliferation rate in specific areas of the regenerating tail.

a) The spinal cord regenerates with the formation of the neural ampulla as a result of the ependymal cell layer proliferation. This is shown by the intense positive signal with the immunostaining against BrdU and PCNA just in this layer of the spinal cord. The high proliferation rate at the base of the neural ampulla probably accounts for thw formation of this transient swollen structure.

b) The notochord shows high proliferation rate in the outer sheath region and in the tip of the regenerating notochord.. The high proportion of BrdU and PCNA positive cells in the conical mass of cells at the tip of the regenerating notochord indicates an area of very active growth. This tip region is composed of cells that have not yet formed their characteristic vacuoles

although the low level of labelling in mature notochord suggests that vacuolisation is not incompatible with an active cell cycle.

c) The muscle tissue at early stages of the tail regeneration appeared to degenerate in a wide area close to the amputation surface. The undamaged myofibres showed a positive signal for BrdU and PCNA exclusively in a small population of flat nuclei localised at the edge of the muscle fibre. At late stages of the process the positive nuclei seemed to migrate outside the fibres but remain in their close vicinity. A remarkable number of undifferentiated BrdU and PCNA positive cells, the blastema cells, were localised also around the notochord and the spinal cord, in close proximity to the cut surface.

Even though the pictures from the histological sections are suggestive regarding the cell lineage of the regeneration bud, they do not give any definite information about the mechanism of the formation of the regenerating tissues. We cannot prove from these results that there is no dedifferentiation or transdifferentiation occurring. To do so it will be necessary to examine the cell lineage by labelling the cell populations *in vivo* and analysing their migration and colonisation movements.

IV-Cell lineage of spinal cord and notochord

Investigation of suitable promoters

Ubiquitous promoters

To perform graft experiments in order to specifically label the tail core tissues, it was necessary to choose a suitable promoter that was active, at high level, in all cell types, and that was independent of any specific controls operating during the tail regeneration process. This is because the label must be retained if cells of the regeneration bud de-differentiate by losing their characteristic identity, and if they re-differentiate with a different phenotype. The promoters studied during this research: cytoskeletal actin promoter (CSKA); elongation factor 1α (EF1α); and Simian cytomegalovirus early promoter (CMV), were chosen based on previous reports as ubiquitous enhancers of GFP expression (Kroll and Amaya, 1993; Amaya and Kroll, 1999). The panel in Fig.IV1 shows examples of transgenic embryo and larvae for GFP expression driven by the three different promoters above described. On the top (Fig.IV1A) a transgenic embryo at stage 27 NF for CSKA-GFP is shown. The GFP used contains a nuclear localization signal, and so tends to concentrate in nuclei but some fluorescence is also visible in the cell cytoplasm. This promoter demonstrates a good GFP expression in whole embryo during the embryonic stages, but nevertheless the intensity of the expression decreases radically during feeding stages (st. 45 NF; data not shown).

Fig.IV1

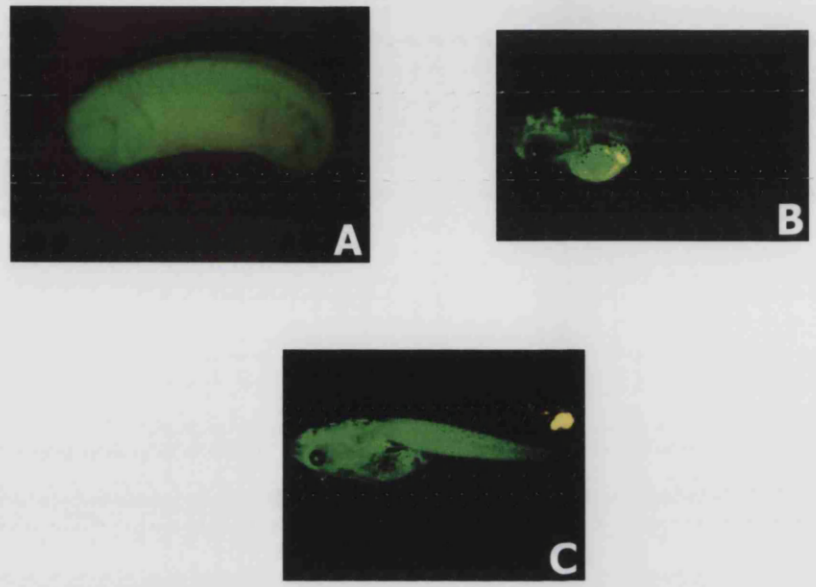


Fig.IV2

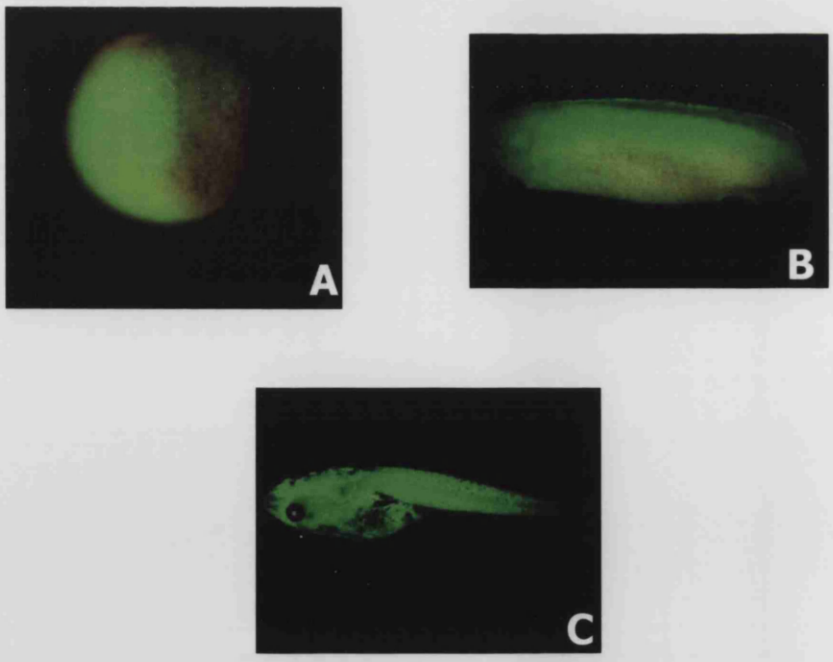


Fig.IV1 Ubiquitous *Xenopus* transgenic embryo **(A)** and larvae **(B,C)** expressing GFP all over. **(A)** Citoskeletal actin (CSKA) promoter driving GFP at stage 27 NF. **(B)** Elongation factor 1α (EF 1α) promoter driving GFP at stage 49 NF. **(C)** Simian cytomegalovirus early (*CMV*) promoter dricing GFP at stage 49 NF.

Fig.IV2 GFP expression during the development of transgenic *Xenopus laevis* for *CMV*-GFP: **(A)** stage 13 NF, **(B)** stage 28 NF and **(C)** stage 49 NF. The GFP keep being expressed in all the development stages under *CMV* promoter.

Probably this decrease of GFP expression is due to a down-regulation of the CSKA promoter when the embryos reach the feeding stages, associated with an up-regulation of other genes involved in nutrition and the new organisation of the digestive system. In the middle and bottom (Fig. IV1B,C) two transgenic tadpoles (st. 49 NF), respectively for EF1α and CMV-GFP are reported. Both the promoters drive the GFP expression ubiquitously in whole animal, but is clear that the CMV promoter shows stronger expression compared with EF1α, and the GFP signal is well retained also during later stages. This is further shown in Fig. IV2 where a panel of embryonic and larval development stages shows the retention of the fluorescent GFP signal, driven by CMV promoter, in all embryonic and larval tissue types until later tadpole stages (st. 49/50 NF) when the tail will be removed in the experiments to be described. The CMV promoter showed a good activity with characteristics matching the demand, nevertheless it was necessary also to check ubiquitous expression in the regenerating tail tissues. In Fig.IV3 the normal time course of the tail regeneration process is presented for CMV-GFP transgenic tadpoles, expressing GFP all over.

Fig.IV3

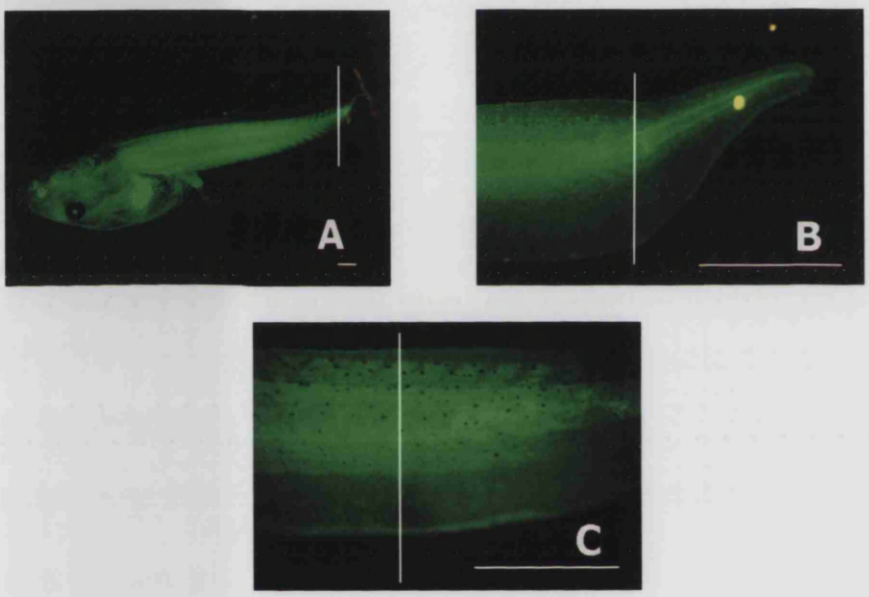


Fig.IV3 Transgenic tadpoles expressing GFP under control of *CMV* promoter. **(A)** Five days after tail amputation, the regenerate keep expressing GFP. **(B)** Ten-day regenerating tissue. **(C)** Complete tail regeneration, all the reformed tissues still express GFP. White line indicates amputation level. Scale bars: 500 μm. (Gargioli and Slack, 2004).

The signal and intensity of the fluorescent expression was retained in the regenerating tissue; all the tail core tissues (the neural tube, the notochord and the somites) were clearly positive for the GFP expression, in the stump and in the regenerate. At five days after surgical tail removal (Fig. IV3A), there is a very intense fluorescent signal in the tail tip. This is because the high cell proliferation rate in the regeneration bud is associated with an increase in GFP expression in those cells. Following, the next days, all the tail re-forming tissues complete differentiation and the regenerating tissues keep expressing the GFP throughout (Fig.IV3B,C). Therefore the CMV promoter was chosen as a good ubiquitous promoter suitable for following transgenic tissue grafts to label specific target tissues of the tail.

Tissue specific promoters

In order to make and compare specific tissue labelling, also some suitable tissue specific promoters were investigated. In particular three promoters were tested by analysing the localisation of the GFP expression: NβTub-GFP (Neural beta Tubulin: spinal cord), pColII-nucGFP2 (Collagen type II: larval notochord) and pCar-GFP (Cardiac actin: muscle) (Fig.IV4). The activity of two of those promoters: NβTub and pCar, was already known (Fig.IV4A-D); however, the activity of the ColII promoter was not known.

Fig.IV4

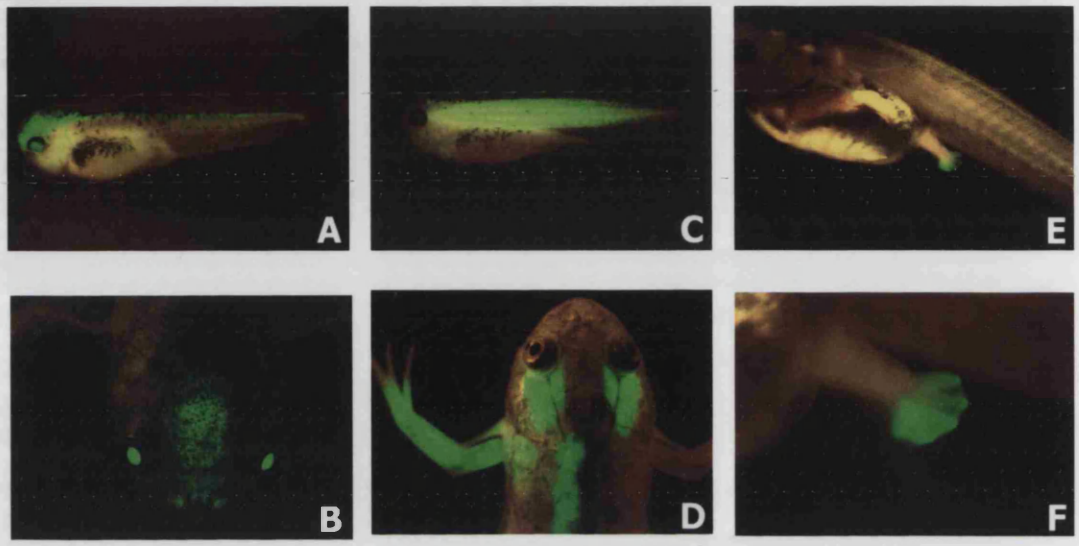


Fig.IV5

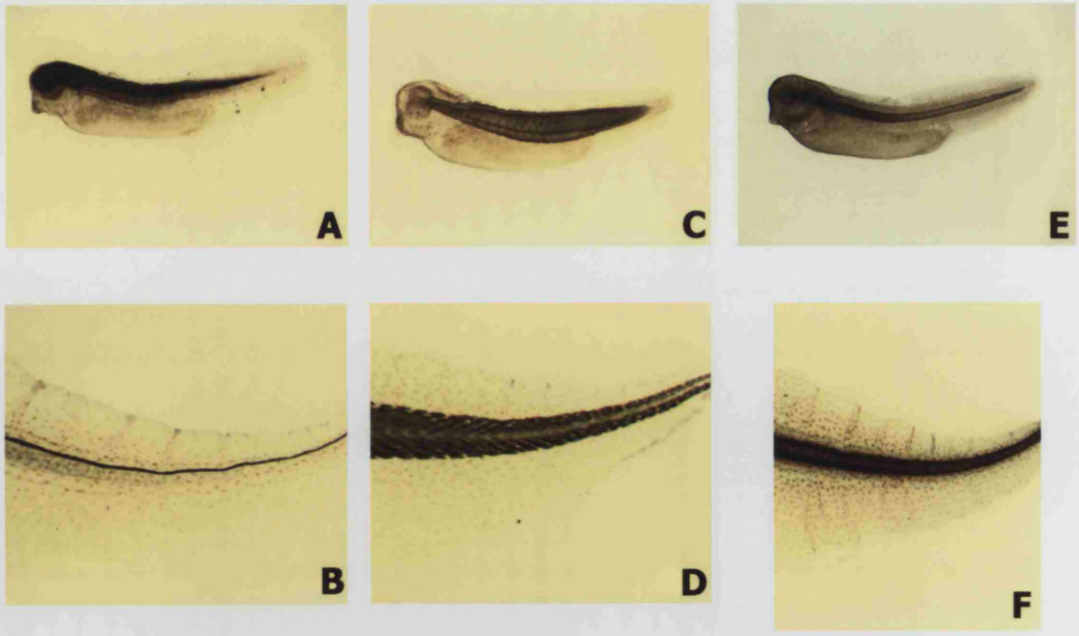


Fig.IV4 Transgenic tadpoles expressing GFP under tissue specific promoter: **(A,B)** Neural β tubulin promoter driving GFP in the neural tissue respectively at stage 38 NF and at climax stage; **(C,D)** Cardiac actin promoter driving GFP in the skeletal muscles respectively at stage 36 NF abd at climax stage; **(E,F)** Type II collagen. driving GFP in the limb cartilage respectively low and high power magnification. The green fluorescent signal is specifically expressed in the tissue aimed.

Fig.IV5 Whole mount immunostaining on embryo stages 28/29 NF **(A,C,E)** and on tail of stage 49 NF larvae **(B,D,F)**. **(A,B)** 2G9 monoclonal antibody (mAb) against neural tissue. **(C,D)** 12/101 mAb against skeletal muscle tissue. **(E)** Mz15 mAb against embryonic notochord, **(F)** anti collagen II mAb against larval notochord.

It turns out that it drives the GFP just in the cartilage of the limbs as shown in Fig.IV4E,F, and not in the notochord. The type II collagen promoter was investigated because in whole mount immunostaining an antibody against ColII (ICN) showed good reactivity by labelling the notochord. There is not a satisfactory molecular marker for the *Xenopus* tadpole notochord, despite the embryonic labelled by MZ15 mAb (monoclonalantibody) against keratan sulphate (Craig et al., 1987). In the panel in Fig.IV5 are presented whole mount immunostaining showing the activity of different specific mAbs reacting against individual tissue at embryonic and larval stages. 2G9 mAb (Jones and Woodland, 1989) directed against differentiated neural embryonic and larval tissue (Fig.IV5A-B); 12/101 mAb (Kintner and Brockes, 1985) directed against embryonic and larval skeletal muscle (Fig.IV5C-D) , and MZ15 mAb (Craig et al., 1987) directed against embryonic notochord (Fig.IV5E) and ColII mAb (ICN) against type II collagene reacting with the larval notochord (Fig.IV5 F). Therefore in the case of the spinal cord and muscle the immuno-labelling is confined to the same tail structure labelled with the fluorescence driven by

the specific promoter. On the other hand the ColII promoter, shows a completely different activity compared to the presence of collagen II protein, driving the GFP in the cartilage-forming areas of the developing limbs. This curious promoter behaviour does not relate to the main topic of the thesis and so was not further investigated. Probably the peculiar behaviour of ColII promoter is due to the fact that is a single enhancer for lima cartilage, and that the whole regulatory region would also contain notochord enhancer.

Grafting technique

Grafting experiments were conducted on neurula stage embryos, at stage 13 or 17 NF. Transgenic embryos expressing GFP in all tissue types, were surgically operated in order to take exclusively the tail tissue required and then to implant this tissue in the same position in a new normal host embryo. Thus explants of transgenic neural plate, notochord or presomite mesoderm (PSM) positives for GFP expression, were transplanted into non-transgenic host embryos in the presumptive tail region, next to the blastopore (Table IV1, Fig.IV6) (Tucker and Slack, 1995a). The neurula stage was selected for these operations because it is possible, with the use of trypsin as described in the Materials and methods, to obtain a very clean separation of tissue layers and thereby obtain pure grafts with the label confined to a single tissue. 24 hour after the transplantation the GFP fluorescence was visible, localised specifically in the position of the grafted tissue (Fig.IV7A-C). Sections immunostained with a commercial antibody against GFP (Materials and methods) showed that GFP was confined to the tissue grafted (Fig.IV7G-I).

Fig.IV6

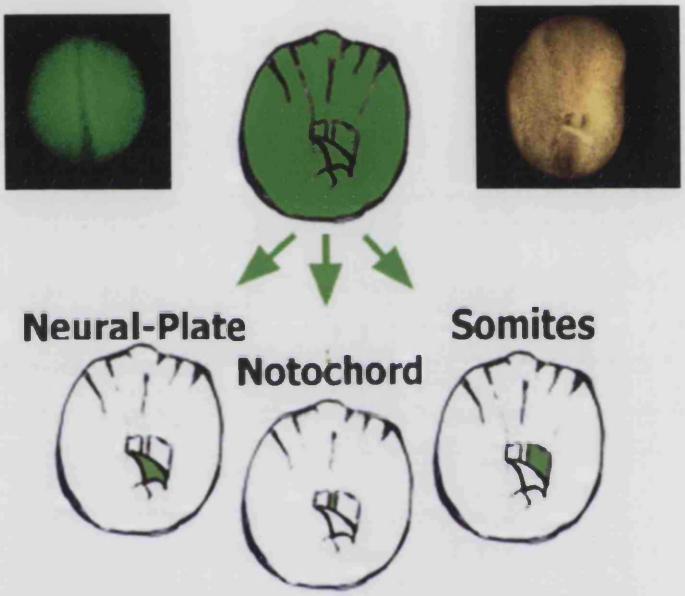


Fig.IV6 The grafting technique for the three tail main tissues performed at neurula stage (stage 13-15 NF). **(A)** *CMV-GFP* donor embryo. **(B)** Normal host showing retraction of posterior neural plate section (Gargioli and Slack, 2004).

Table IV1. Summary of cell population labelling experiments

Graft	Specific label in one tissue	Tissue regeneration labelled		
	Cases	Spinal cord	Notochord	Muscle
Neural Plate	12	12	0	0
Notochord	13	0	13	0

At stage 48-49 NF, the stage of feeding tadpole, the specificity of the labelling was extremely obvious. Because of the transparency of the tadpole tail it was possible to visualise the fluorescent cells at single cell resolution *in vivo* and confirm that the expression of the GFP was limited to the tissue originally transplanted, with no fluorescence signal in other cells outside the targeted tissue (Fig.IV7D-F). The labelling patterns of the grafts are very similar to the monoclonal antibody staining results for spinal cord and notochord (Fig.IV5B, F). It should be noted that the neural graft took tissue from the central part of the neural plate which ends up in the ventral half of the neural tube. The dorsal half of the tube is not labelled and so I should not expect to see, and did not see, any migratory neural crest cells labelled. Individual myofibres either had all nuclei labelled or no nuclei labelled. This is because if some myoblasts contributing to a fibre carry the nucGFP gene then the protein will be synthesized in the fibre and will enter all of the nuclei.

g.IV7

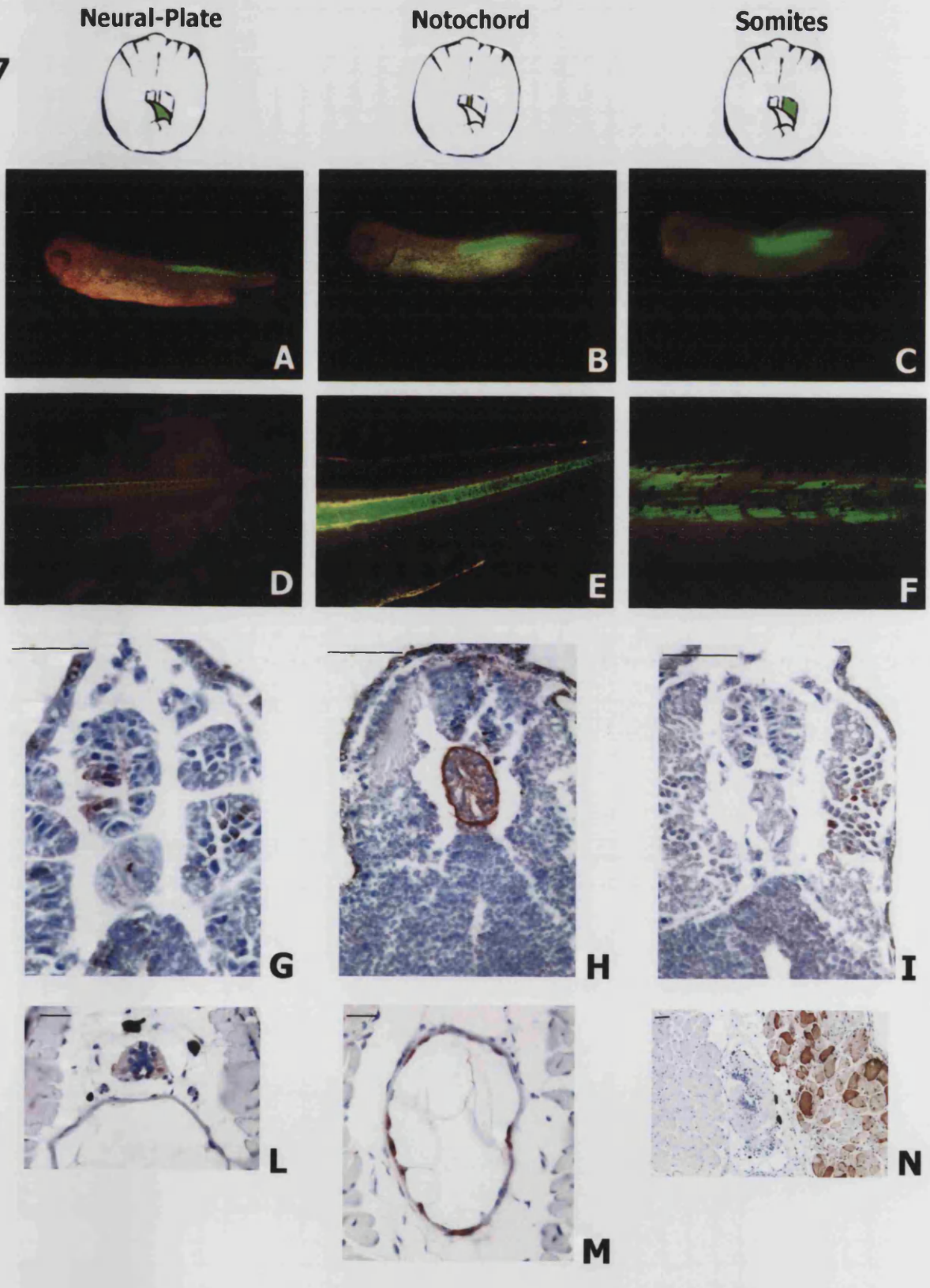


Fig.IV7 GFP-labelled grafts of the three embryonic tissues to the presumptive tail region. **(A-C)** Embryo stage 28/29 NF. **(D-F)** Tadpole stage 49 NF. **(G-I)** Transverse sections of caudal region of host and tailbud embryo stage 28 NF. **(L-N)** Sections of tail at tadpoles stage 49 NF. The sections of the three different graft types are immunostained with anti GFP (red/brown) and counterstained with Haematoxylin (blue) showing specific labelling of each of the three tissue types. Scale bars: 100 μm.

It has previously been shown that the first formed myofibres in *Xenopus* tadpoles are mononuclear, but that they become multinucleate over the stage range 40-46 NF (Muntz, 1975). Again the results of the *in vivo* examination were confirmed by sectioning and immunostaining for GFP (Fig.IV7L-G): the pictures present the effective accuracy of the graft technique showing the specific immunodetected localization of the GFP in the tissues targeted for the transplantation and then the accuracy of the grafting technique and the specificity of the labelling.

Study of label in regenerates

The transplanted *Xenopus* tadpoles, labelled in one of the three tail core tissues, were allowed to reach stage 49-50 NF and were then amputated at 50% of the tail length. The tail regeneration process was analysed and followed *in vivo* by anaesthetising the larvae at regular intervals after the surgical amputation and taking pictures under a stereomicroscope equipped with UV lamp and GFP filter, as described in the Materials and methods.

Spinal cord

At 5 days after tail amputation, the regenerating spinal cord was already visible and was in continuity with that of the stump (Fig.IV8A). The blastema

surrounding the re-forming spinal cord was completely negative for the GFP fluorescence, as was the notochord. The labelled cells expressing GFP were localised only in the regenerating spinal cord, all other surrounding tissue cells being completely negative. In the following days the regenerating spinal cord kept elongating, always in continuity with that of the stump (Fig.IV8B,C). The blastema was not involved at all in the formation of the new spinal cord, the stump spinal cord drives fully the regenerative process. Once the stage of complete tail regeneration was reached, the GFP was still exclusively and specifically expressed in the spinal cord as in it had been in the original tail.

Notochord

The regenerating notochord behaved in a similar manner to the spinal cord. After 5 days the re-forming notochord was already visible in continuity with that of the stump. The blastema around the regenerating notochord was negative for the expression of the GFP (Fig.IV8D). The labelled cells, as in spinal cord regeneration, were not visible outside the notochord itself. With the elongation of the regenerating notochord the fluorescent labelling remained confined to the notochord, and all the other tail tissues were still completely negative (Fig.IV8E,F). When full regeneration was completed, the expression of the GFP was always localised exclusively and specifically in the notochord as in the original tail.

Fig.IV8

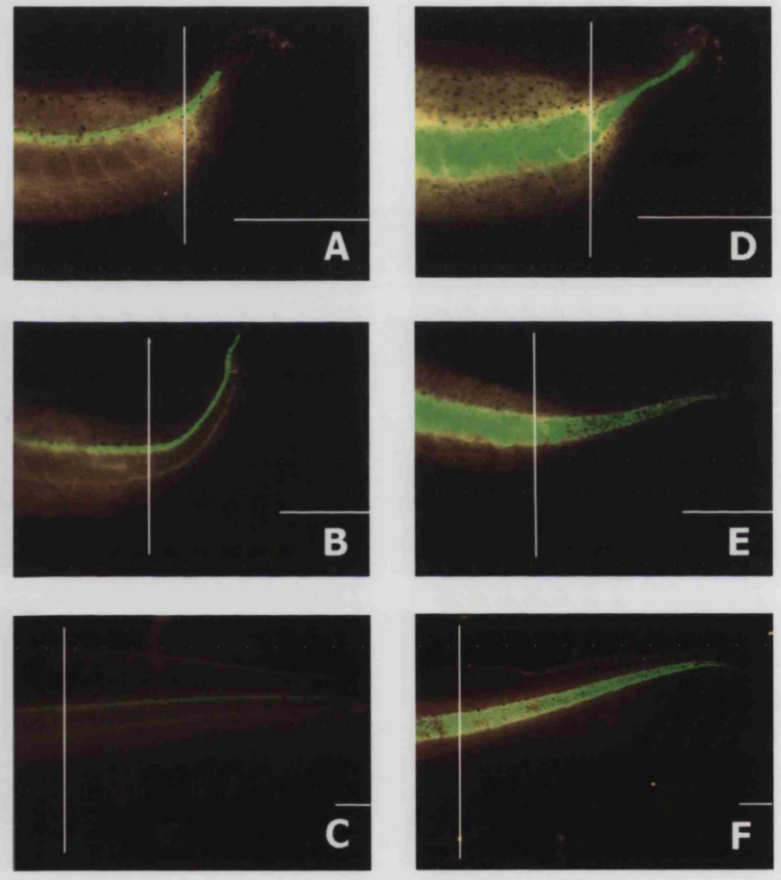
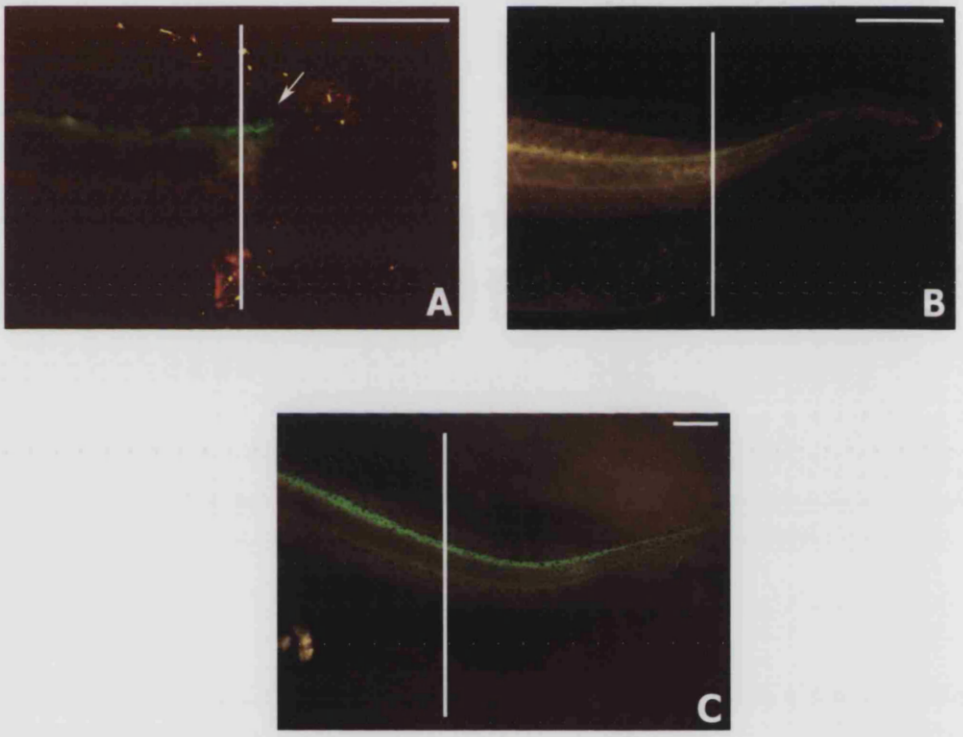


Fig.IV8 **(A-C)** Spinal cord labelled regenerating tadpole tails, showing localisation of the GFP exclusively in the spinal cord of the stump and the regenerate. **(A)** Five days after amputation. **(B)** Ten days after amputation. **(C)** Twenty days after amputation. **(D-F)** Notochord labelled regenerating tadpole tails showing localization of the GFP exclusively in the notochord of the stump and the regenerate. **(D)** Five days post-amputation. **(E)** Ten days post-amputation. **(F)** Twenty days post-amputation. With bars indicates the amputation level. Scale bars: 500 μm. (Gargioli and Slack, 2004).

Conclusion

By considering the results described in this chapter and the evident and clear behaviour of the spinal cord and the notochord, it is possible to reach a firm conclusion based on the GFP expression in these tissues. The evidence in this section, shows the presence and persistence of the fluorescent green expression in the tail tissues labelled by transplantation: spinal cord and notochord. Segments of those tissues were taken from the tail presumptive region (close to the blastopore) of transgenic embryos at neurula stage (st. 13 NF), expressing GFP ubiquitously in all embryonic tissues driven by CMV promoter. Then they were transplanted in the same region of normal host embryos, showing the effective functionality and specificity of the grafting technique to label target tail core tissues. The specific fluorescent labelling was also confirmed by immunostaining, detecting the GFP signal exclusively in the tissue transplanted. During the tail regenerative process in *Xenopus* tadpoles, it is clear that the GFP expression retain specific localization in the tissues targeted and nowhere else. By labelling the spinal cord, during regeneration just the same re-forming tissue presented the GFP expression. All the other regenerating tissues of the regeneration bud and the blastema were completely negative for the fluorescent signal, the fluorescent stump

Fig.IV9



spinal cord was in continuous with the fluorescent regenerating cord. The notochord presents identical behaviour: the only positive tissue expressing GFP was the regenerating notochord itself. The tissues surrounding the notochord did not present any positive fluorescent signal.

The labelling of these two major tissues gives a clear answer to the question about their origin in regeneration. For spinal cord and notochord there is no metaplasia. The spinal cord arises from the spinal cord of the stump and the notochord from the notochord of the stump. A further piece of evidence for the neural origin of the regenerating spinal cord is given by the experiments performed with transgenic tadpoles expressing GFP under NβTub. In Fig.IV9 is shown a panel with the normal time course for the tail regeneration. The GFP is expressed specifically in the spinal cord such as in the graft experiments and obviously is re-expressed in the regenerating cord re-differentiating in new spinal cord. But at very early stage, 3 days post amputation, it is interesting to note in the regeneration bud the green fluorescent labelling of a swollen structure strictly resembling the neural ampulla (Fig.IV9A). This demonstrates again the origin of the regenerating spinal cord from the stump cord.

Fig.IV9 Regenerating spinal cord specifically expressing GFP under Neural β tubulin promoter showing localisation of the GFP expression exclusively in the neural tissue also at early regenerating stages. **(A)** Three days after tail removal showing GFP positive neural ampulla (white arrow). **(B)** Seven days after tail removal. **(C)** Ten days after tail removal. White bar indicates the amputation level. Scale bars: 500 μm.

V-Cell lineage of muscle

Car-Cre/Lox labelling compared to *Car-GFP*

In order to set limits to the origin of the regenerated muscles, it is convenient to describe the experimental route followed during this research leading up to the results from the grafts of presomite mesoderm (PSM). First is an experiment based on the *Cre/Lox* system, where a proportion of myofibres were permanently labelled with the expression of GFP following an intra-chromosomal recombination to remove a translational stop signal. To avoid translation starting in absence of the recombinase *Cre*, an 800 base pairs *STOP* sequence series was placed between the *Lox* sites between the CMV promoter and the GFP coding sequence, as described in Materials and Methods. The sperm incorporating this plasmid DNAs was injected into unfertilized eggs in order to make them transgenic. I should not have expected to see, and did not see, any fluorescent cells expressing GFP in the embryos and tadpoles resulting from this experiment, proving the effective functionality of the *STOP* sequence.

The Cre-lox experiment was conducted in founder tadpoles, not by crossing *Cre*-expressing and reporter line animal together. The *Cre* was driven by the muscle-specific cardiac actin promoter (*Car*) and the reporter plasmid, as described above, containing CMV promoter driving GFP with a *Lox-STOP-Lox*

sequence at the beginning of the GFP coding region. The effect is to label permanently with GFP a proportion of the cells that have activated the *Car* promoter. These cells will express GFP under control of the *CMV* promoter. So if they de-differentiate, losing the identity of muscle cells and the *Car* promoter, it will be possible to follow their fate of as the CMV-GFP will remain active. These specimens were compared to others which were simply transgenic for *Car*-GFP, which will show green fluorescent label in all cells which currently have the *Car* promoter active: i.e. all the cells differentiated as muscle cells. In Car-GFP tadpoles just and exclusively the new re-forming myofibres, differentiating in the regenerative process will express GFP.

Ten transgenic tadpoles expressing *Car-Cre* and CMV-*Lox-STOP-Lox*-GFP were created with a sufficient proportion of myofibres labelled to make the experiment possible (Fig.V1A,B,E,F). Unlike the *Car*-GFP transgenic *Xenopus* larvae, only some of the myotome cells early in the somite, and later in the muscle myofibres are labelled. But the proportion gradually increases with time because of new recombination events occurring where the *Cre* recombinase is expressed. This delay is due to the fact that the homologue recombinase must scan the whole genome searching for the *Lox* sites to recombine. Three days post amputation, the regeneration bud contained no fluorescent cells at all for either of the two transgenic models: *Car-Cre* and *Car*-GFP (Fig.V1C,G). This shows that there is no de-differentiation of multinucleate fibres to mononuclear cells. In the *Cre/Lox* transgenic regenerating tadpoles, any cells de-differentiated from green fibres would still be making GFP.

Fig.V1

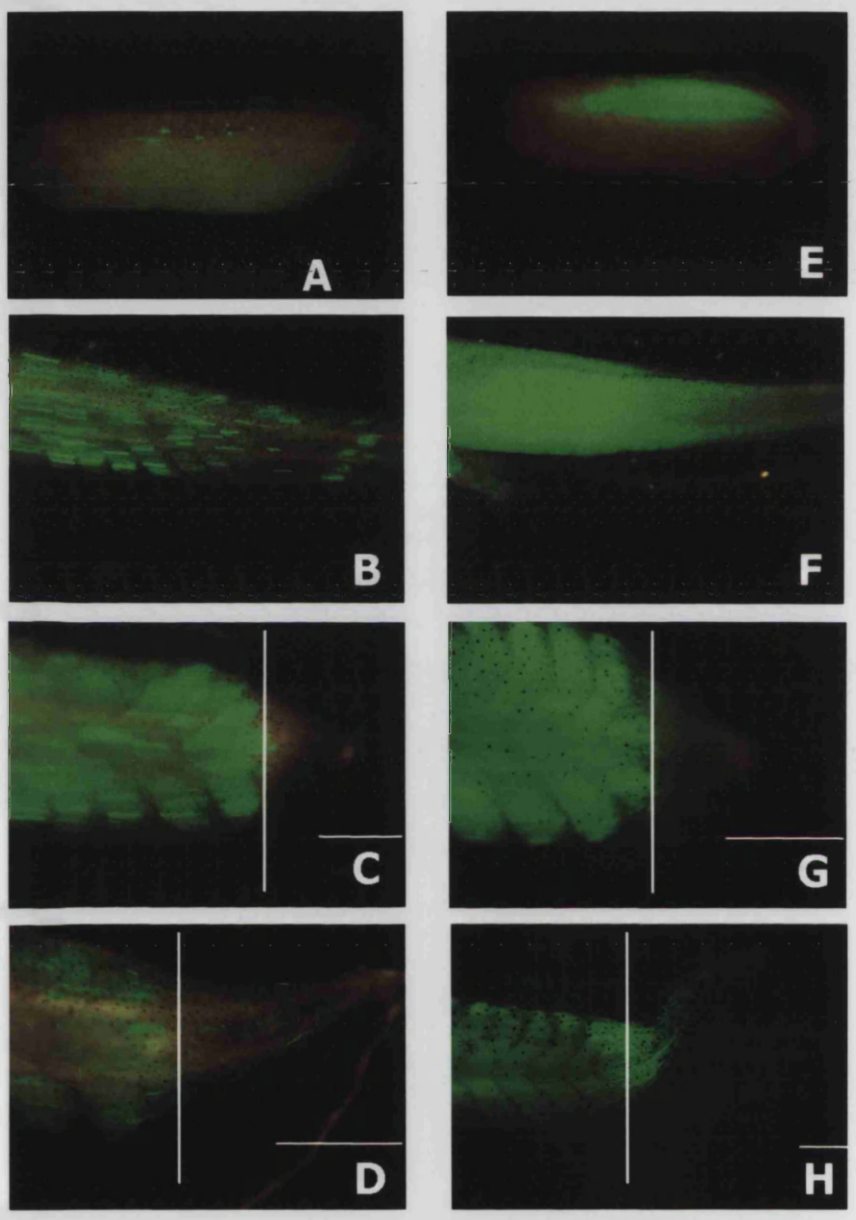


Fig.V1 (A-D) *Car-Cre/Lox-GFP* transgenics expressing GFP in the myofibres after intrachromosomal recombination. **(E-H)** *Car-GFP* transgenics expressing GFP in all myofibres. **(A)** Stage 28 NF embryo showing a few somatic cells starting to express GFP. **(B)** Stage 49 NF tadpole, before tail amputation, expressing GFP in numerous myofibres. **(C)** Regenerating tail 3 days after amputation showing no GFP labelled cells in the undifferentiated blastema. **(D)** Five day regenerating tail starting to express GFP in a few new formed myhofibres. **(E,F)** Stage 28 NF embryo and stage 49 NF tadpole expressing GFP in the muscle under control of the cardiac actin promoter (*Car*). **(G)** Three day regenerating tail showing no GFP labelled cells in the undifferentiated blastema. **(H)** Five day regenerating tail showing substantial new formation of labelled myofibres. White bar indicates amputation level. Scale bars: 200 μm. (Gargioli and Slack, 2004).

In the *Car*-GFP transgenic larvae, although the production of GFP would cease on de-differentiation, the protein itself should persist for a few days. After 5 days from the tail removal a substantial number of green myofibres appeared in the regenerated region of the *Car*-GFP transgenic (Fig.V1H). This indicates the normal tempo of reforming differentiated muscle during the regeneration process. By contrast in the *Cre/lox* transgenics, there were just a few isolated green fibres (Fig.V1D), believed to be the result of new *Cre* recombination events. This is not surprising because the cardiac actin promoter will now be active again, as the *Cre* recombinase will be expressed in all myofibres, regardless of their origin. In fact the time scale and position of appearance of the few fluorescent fibres seen in the regenerating tail of the *Cre/lox* transgenic system indicates that they are not due to de- and redifferentiation of muscle cells previously expressing GFP. The absence of any short term labelling of mononuclear cells in the regeneration bud, and the slow appearance of the labelled myofibres in the *Cre/lox* tadpoles suggests that the muscle fibres themselves contribute no cells to the regeneration bud. This

is consistent with the morphological picture, which shows large scale degeneration of myofibres in the vicinity of the cut surface, and it suggests that the new muscle must come from some other cellular source than pre-existing myofibres.

LDL-diI endothelial labelling

Because of evidence showing no contribution of myofibres to the regenerate, the possibility of different sources of cells has been considered; in particular the contribution from other mesodermal cell populations to the regeneration bud. One possibility is the mesoangioblasts that are thought to be associated with the early vascular system, and which have been shown to contribute to muscle renewing and restoration in mammalian models (De Angelis et al., 1999; Sampaolesi et al., 2003). In order to investigate this cell population the procedure of Levine et al. (2003) was used to label the entire circulatory system, in particular the endothelial cells, of the tadpole by injection of LDL-diI in the heart ventricular cavity (Materials and Methods). This gives a clear labelling of the blood vessels and circulating cells, and should label any pluripotent cells associated with the circulatory system. If any cells in the regeneration bud arose from the endothelial cells or circulating cells labelled originally then this should be apparent from the red fluorescence. 20 tadpoles were treated as above described and their tails amputated, but the regenerates did not show any label in the regeneration bud or in the new myofibres (Fig.V2). Therefore it is unlikely that the re-forming muscle arises

from haematopoietic cells or angioblasts. Thus other cell populations needed to be analysed in order to investigate the tail regenerating muscle origin.

Skin graft for dermal cell labelling

In the light of the negative results regarding haemo- and mesoangioblast contribution to the formation of the regeneration bud and the re-forming myofibres, the dermis was considered as next relevant mesodermal candidate tissue in order to investigate the regenerating muscle origin. To address this question skin grafts were performed to label specifically the dermal cells. The epidermis is in a close and strong contact with the dermis, therefore by transplanting a segment of skin inevitably is grafted the respective underlying segment of dermis. The graft will of course also contain epidermis, but previous evidence makes it very unlikely that this would be a precursor of muscle cells. Using the same transgenic technique as described in Chapter IV, segments of tail skin were removed from tadpoles at stage 48 NF expressing GFP ubiquitously (under the *CMV* promoter) and transplanted in the same position of non-transgenic normal host tadpoles from which a region of the skin was peeled off (Fig.V3A). The host larvae were allowed to heal for 2 days and then the tail was removed at the level of the skin implantation to analyse the destiny of the labelled dermis cells. After 3 days from the tail removal a substantial number of green fluorescent mononucleated cells are visible on the regeneration bud, indicating the migration of dermis cells through the regeneration bud blastema. Five days post-amputation, the amount of mononucleated GFP positive cells increases proportionally with the growth of

Fig.V2

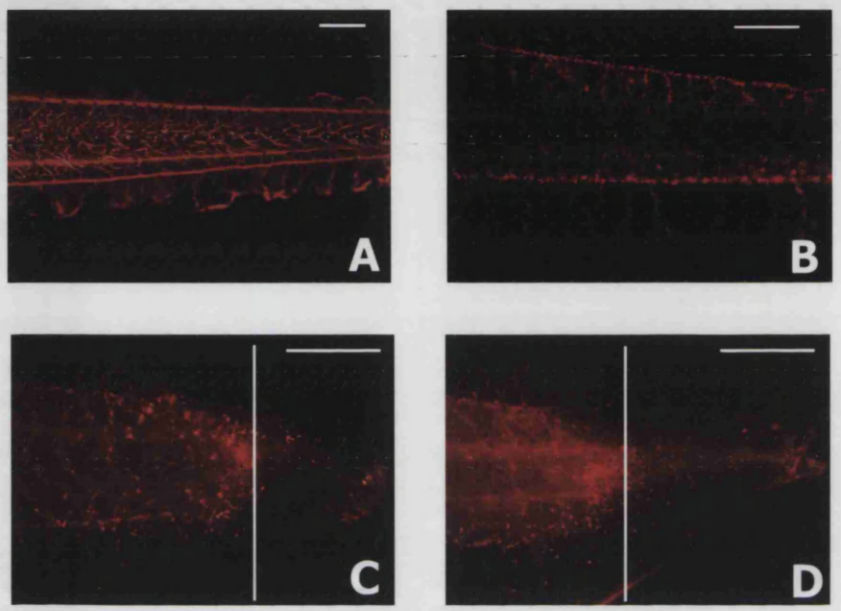
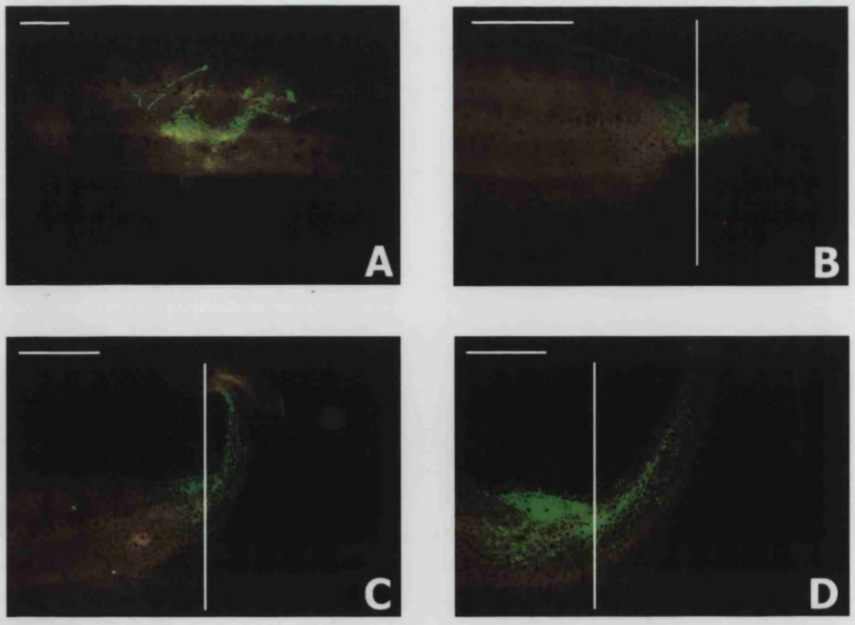


Fig.V3



the regenerating tail, due to the intense cellular proliferation of the regeneration bud (Fig.V3B,C). Nevertheless the regeneration bud at this stage contained no green fluorescent cells which were elongated or fusing in the manner of myofibres. At 10 days after tail removal the regenerating tail showed a considerable amount of GFP positive mononuclear cells, indicating the high rate of cell proliferation of the dermis during the tail regeneration process (Fig.V3D). But all the fluorescent cells were still mononuclear, indicating no transdifferentiation to muscle cells. Therefore these results indicates that the dermis contributes to the formation of the regeneration bud blastema, but does not give rise to myofibres in the regenerate. There is also no obvious contribution to the notochord or spinal cord of the regenerate so the dermal cells appear to retain their tissue type identity even though they participate in blastema formation. Once again this is consistent with the morphological analysis using simple histology.

Fig.V2 LDL-diI injected larvae at stage 49 NF showing a red fluorescent signal in the whole circulatory system just after the ventricle injection **(A)** and 24 hours later **(B)**. **(C)** Regenerating tail at 3 days after tail removal showing no red labelled cells in the regeneration bud. **(D)** Five days post amputation showing the regeneration bud and the reforming muscles still being negative for red fluorescent signal. White bar indicates amputation level. Scale bars: 200 μm.

Fig.V3 Stage 49 NF tadpole tail grafted with transgenic skin segment expressing GFP under *CMV* promoter. **(A)** Twenty-four hours post transplantation showing GFP expression of the derma cells in the host tail. **(B)** Three days after tail removal showing the regeneration bud starting to be positive for the green fluorescence. **(C,D)** Five and ten days after tail removal showing the proliferation of the GFP positive derma cells colonising the regenerate. The GFP positive derma cells from the transplanted skin contribute to the undifferentiated blastema formation but do not show cell fusion in order to form myofibres. White bar indicates amputation level. Scale bars: 200 μm.

Fig.V4

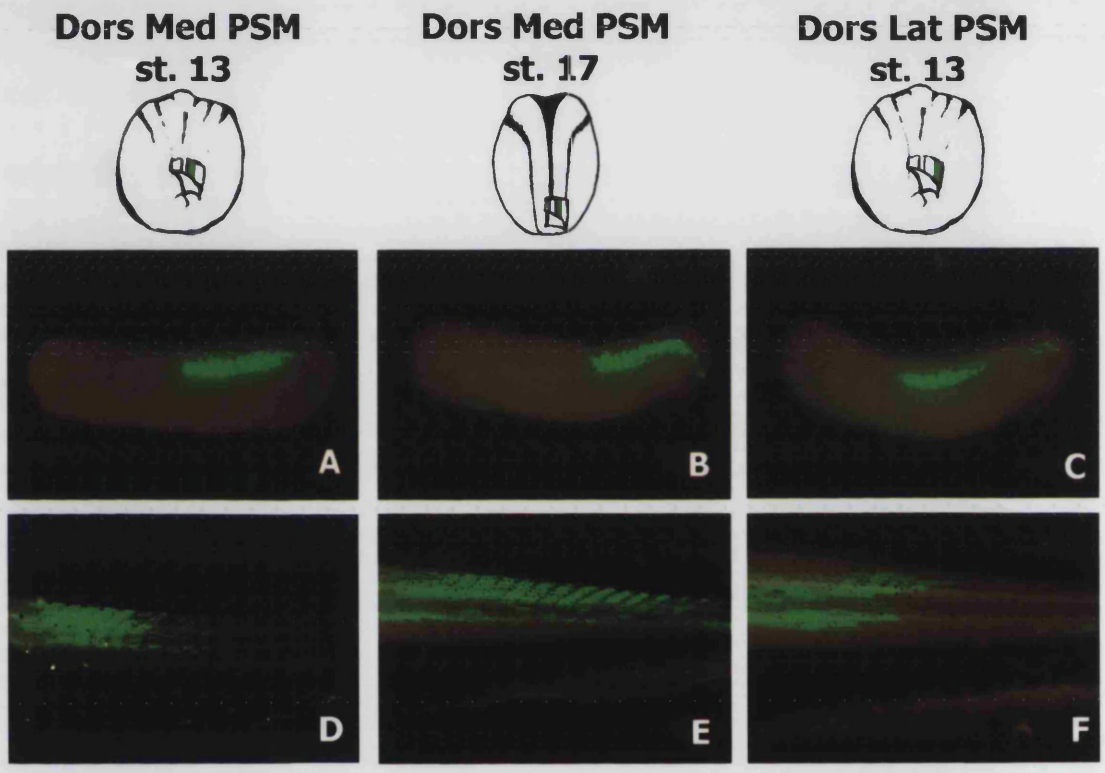


Fig.V4 Presomite mesoderm (PSM) grafts from different stages and positions. In all cases, GFP expression is visible in the somites then in the myotomes. **(A-C)** Transplanted embryos at stage 28 NF. **(D-F)** Transplanted larvae at stage 49 NF. (Gargioli and Slack, 2004).

Presomite plate grafts

The most informative data relating to this question of muscle origin was that from the grafts of neurula stage presomite mesoderm (PSM), although the behaviour was somewhat more complex that that of neural plate and notochord grafts as it depends on the stage of the donor and on the position of origin of the graft within the PSM. Three different types of graft were performed:

PSM adjacent to the notochord at st. 13 NF (early medial)

PSM adjacent to the notochord at st. 17 NF (late medial)

PSM from a more lateral position at st. 13 NF (early lateral)

In all the three cases the labelling of the host tadpoles was exclusively in the somites at embryonic stages and in the myotomes at larval stages (Fig.V4). However, the behaviour during tail regeneration was completely different.

1. For early medial PSM grafts, at 3 and 5 days post-amputation the blastema region was completely negative for GFP expression, even if the adjacent stump muscles showed strong fluorescent labelling (Fig.V5A,B). With the continuation of tail regeneration the re-forming muscle fibres remained negative for GFP expression. No green fluorescence signal was seen in the re-growth tail when it was completing regenerated, except for the previously labelled muscle fibres in the stump (Fig.V5C,D).

2. For the late medial PSM grafts, by 3 days after amputation part of the blastema was already labelled by undifferentiated green mononuclear cells expressing GFP. At 5 days the labelling of the regenerating muscles became wider and more clear. In general following PSM graft only some of the muscle fibres at the level of the amputation are labelled, as there is some mixing of donor-derived with host-derived fibres. The position of the green fluorescent mononuclear GFP positive cells in the blastema was adjacent to the labelled myofibres in the stump and the proportion of labelled to non-labelled cells roughly similar to that in adjacent stump (Fig.V6A). This suggests that the mononuclear cells were migrating from previously labelled muscle tissue to colonise the regenerating tail and give rise to new muscle fibres. After 5 days from tail removal, some GFP positive multinucleate myofibres could be seen (Fig.V6B). In these specimens, no other tissue apart from myofibres showed GFP expression, at 10 and 20 days post-amputation (Fig.V6C,D).
3. For the grafts of early lateral PSM at 3 and 5 days no fluorescent cells were seen in the regenerating blastema (Fig.V7A,B,). Nevertheless by 10 days, some fluorescent myofibres appeared, although fewer and fainter the in the stump (Fig.V7C). Like the late medial PSM graft, the labelled fibres were in continuity with the labelled cells in the stump. The regenerating muscle fibres showed a faint GFP expression compared with that of the stump myofibres. The faintness is presumably due to the fact that only a few fluorescent cells participate in the formation of each myofibre, so the overall GFP intensity within the fibres is diluted. When

Fig.V5

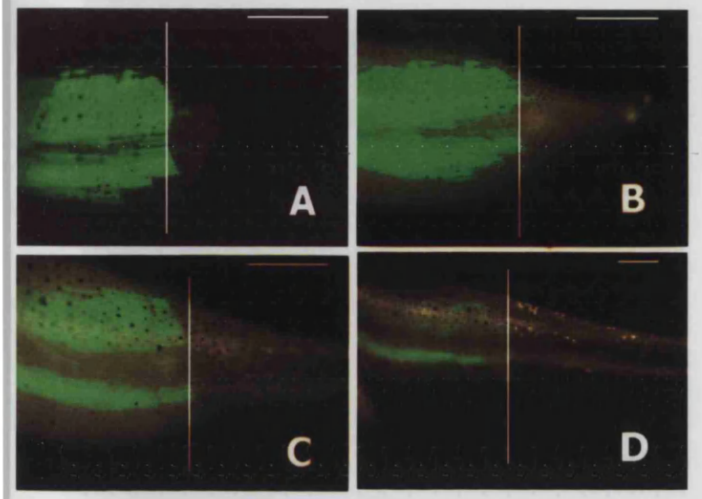


Fig.V6

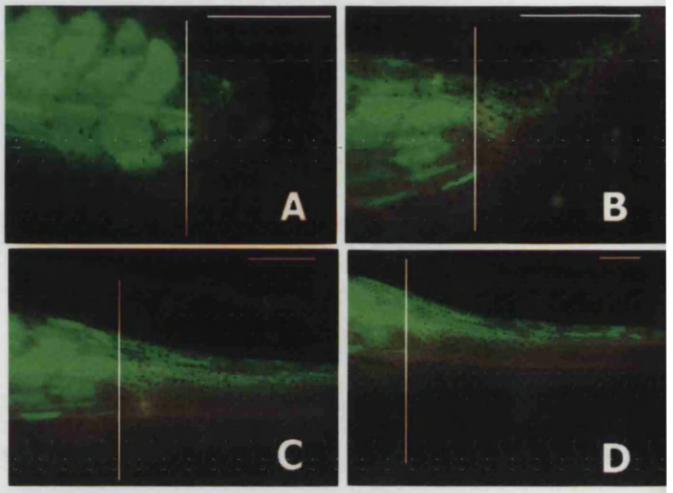
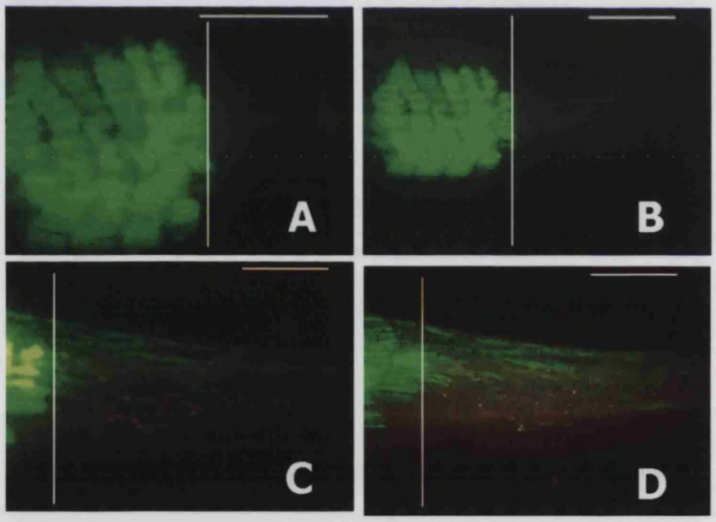


Fig.V7



the tail regeneration was complete, the GFP positive fibres persisted, and no fluorescent label was present in any other tissue except for muscles (Fig.V7D).

The different behaviour of the three types of PSM graft confirms that the presence of labelled myofibres in the stump, regardless of their position in the cross section of the tail, does not necessarily guarantee the formation o any labelled myofibres in the regenerate; experimental results are summarised in table V1. The myofibres must, therefore, originate from some other cell type derived from the presomite mesoderm, and present in the myotomes, whose precursors are found in the lateral rather that th medial PSM. The presence of even more precursors in the late PSM graft is fully consistent with this idea, since there is a massive dorsal convergence of mesoderm during neurulation, so a late medial graft will contain cells that were in a substantially more lateral position at earlier stages.

Fig.V5 Regenerating tadpoles labelled in the myotomes by early medial PSM graft. **(A)** Three days post-amputation. **(B)** Five days post-amputation. **(C)** Ten days post-amputation **(D)** Twenty days post-amputation. There is no labelling of myofibres, or any other cells, in the regenerate. White bar indicates amputation level. Scale bars: 200 μm. (Gargioli and Slack, 2004).

Fig.V6 Regenerating tadpoles labelled in the muscles by late medial PSM graft. **(A)** Thre days post-amputation. **(B)** Five days post-amputation. **(C)** Ten days post-amputation **(D)** Twenty days post-amputation. There is substantial labelling of myofibres in the regenerate. White bar indicates amputation level. Scale bars 200 μm. (Gargioli and Slack, 2004).

Fig.V7 Regenerating tadpoles labelled in the muscles by early lateral PSM graft. **(A)** Three days post-amputation. **(B)** Five days post-amputation. **(C)** Ten days post-amputation **(D)** Twenty days post-amputation. There is slight labelling of myofibres in the regenerate. White bar indicates amputation level. Scale bars 200 μm. (Gargioli and Slack, 2004).

Table V1. Summary of muscle cell population labelling experiments

Graft	Specific tail muscle label	Tissue regeneration labelled		
	Cases	Spinal cord	Notochord	Muscles
Medial PSM st. 13	20	0	0	0
Lateral PSM st. 13	10	0	0	7
Medial PSM st. 17	12	0	0	12
Cre-Lox (3d reg)	10	0	0	0

GFP localization in different muscle cell type

In order to characterise the difference between the three different PSM graft types in term of cell population, the grafted tadpoles were analysed by immunostaining against GFP, as described in Materials and Methods. Both longitudinal and transverse sections were used although the transverse sections proved most informative. The nuclear localisation signal means that GFP protein is concentrated in nuclei, nevertheless highly expressing cells show some in the cytoplasm as well.

1. Early medial PSM graft sections showed the labelling of large nuclei, appearing round in transverse section, within the segmented muscle of the grafted side of the tadpole (Fig.V8A). Those are the nuclei of the muscle fibres, called myonuclei, expressing GFP. In the same sections many GFP negative nuclei, stained blue by the Haematoxylin counterstain, can be seen. Two different types of negative nuclei were visible. The most numerous were the round myonuclei similar to the GFP positive ones,

representing the population of negative muscle fibres, host-derived not expressing GFP. The second type of nucleus showed a different morphology, being flat, placed only in the edges of myofibres, but intimately associated with them.

2. Late medial PSM transplant sections showed a subtly different pattern of labelling. They also showed immunoreaction with the round myonuclei, but here some flat nuclei were also GFP positive (Fig.V8B). Where the muscle fibres showed GFP expression, both types of nuclei tended to be positive. In some cases flat nuclei could be labelled in isolated regions away from the positive myonuclei.
3. Early lateral PSM graft sections immunostained for GFP showed an intermediate appearance. Mostly the GFP positive nuclei were round myonuclei, as in the early medial PSM transplants; nevertheless, a very small number of flat nuclei also showed GFP expression (Fig.V8C). The proportion of GFP positive flat nuclei was much lower than for the late medial PSM grafts.

In the three different types of PSM transplants the proportion of labelled flat nuclei was scored over 100 cells in several GFP positive areas and the results are summarised in table V2. The flat nuclei have the appearance of muscle satellite cells (Mauro, 1961; Muntz, 1975). These are mononuclear cells lying within the basement membrane of the myofibres that can re-enter mitosis and contribute to growth and regeneration of the muscles. A monoclonal antibody (mAb) against the transcription factor Pax7, a molecular marker for

Fig.V8

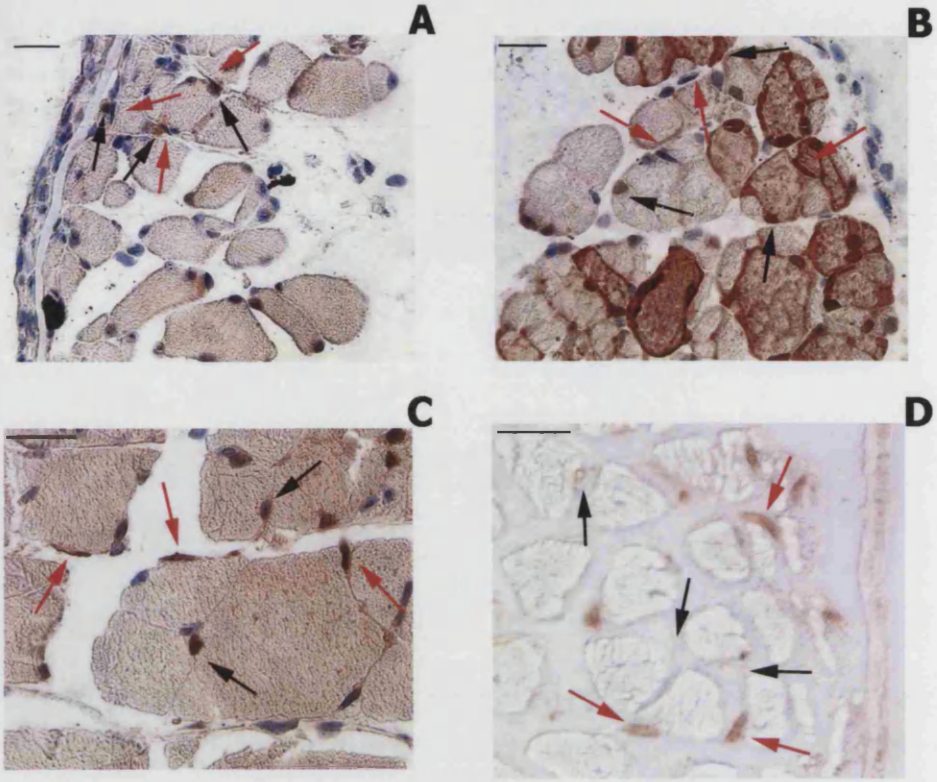
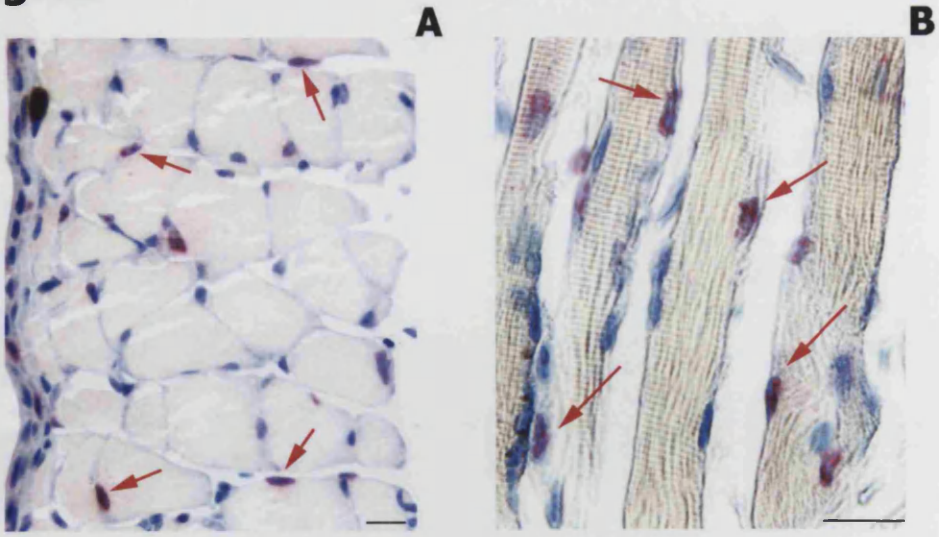


Fig.V9



muscle satellite cells in mammals (Seale, et al., 2000), was used in order to confirm this identification.

The cell type labelled by Pax7 mAb was morphologically identical to the type labelled by GFP immunostaining in the types of graft that give labelled muscle in the regenerates (Fig.V8D). Further proof of the identity of these cells as satellite cells is given by the incorporation of BrdU. It has been demonstrated that the only cell type proliferating in mammalian differentiated muscles are the satellite cells (Walsh and Perlman, 1997). Unamputated tadpoles were injected with BrdU and processed 24 hours later.

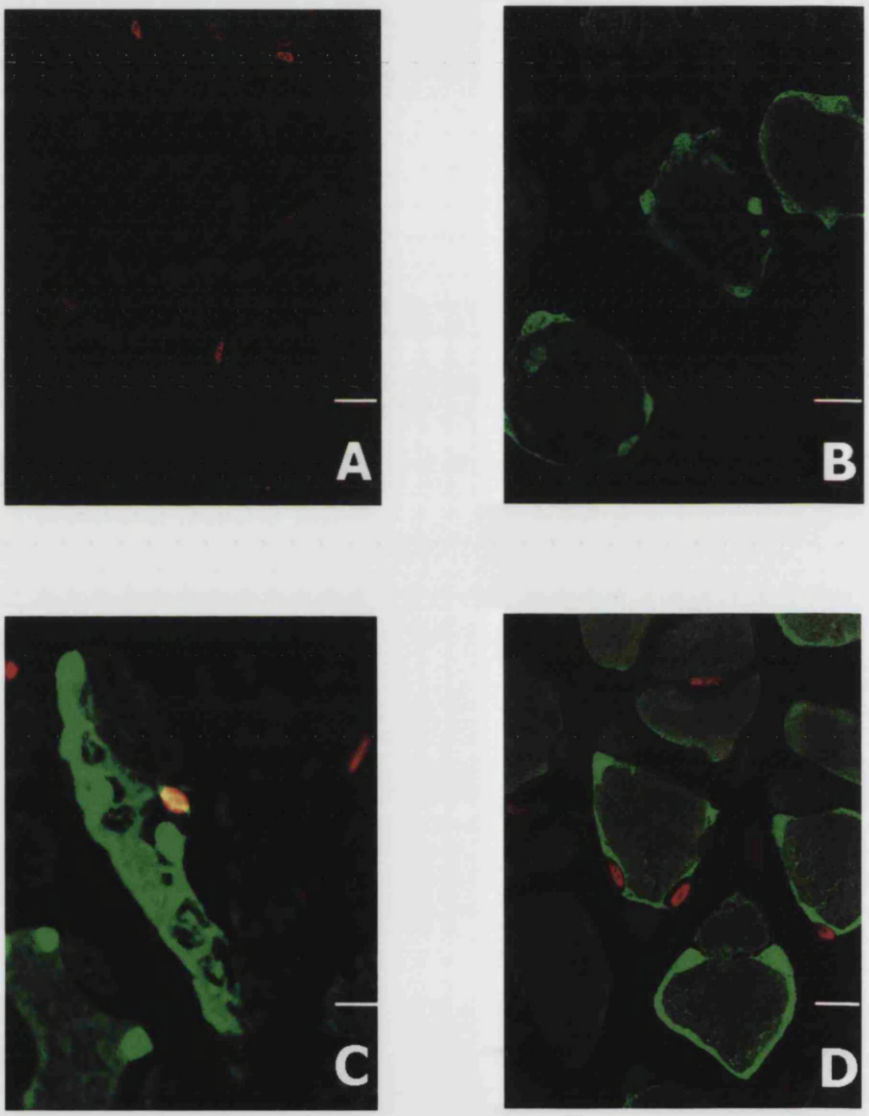
Table V2. Ratio of GFP positive flat nuclei (1000 nuclei were counted)

PSM graft	Early Medial	Late Medial	Early Lateral
%GF flat nuclei	0	58	5

Fig.V8 (A-C) GFP expression in myotomes after PSM grafts performed at different stages and level. Transverse sections were immunostained with antibody against GFP (brown) and counterstained with hematoxylin (blue). **(A)** Early medial PSM grafted muscles expressing GFP in the myonuclei (black arrows), but not in the satellite cell nuclei (red arrows). **(B)** Late medial PSM grafted muscles showing GFP expression in both myonuclei and in satellite cells. **(C)** Early lateral PSM grafted muscle showing GFP expressed in myonuclei and in few satellite cells. **(D)** Muscle transverse section imunostained with Pax7 antibody (brown) (not counterstained). Pax7 expression is detected in the flat nuclei (red arrows) and not in the large round myonuclei (black arrows), suggesting that the flat nuclei are those of satellite cells. Scale bars 10 μm. (Gargioli and Slack, 2004).

Fig.V9 BrdU incorporation in *Xenopus* tadpole muscle shown by immunostaining and counterstained with haematoxylin (BrdU in red). **(A)** Transverse section. **(B)** Longitudinal section. Positive cells are indicated by red arrows, and are mostly the flat nuclei at the edges of myofibres, indicative of satellite cells. Scale bars 5 μm. (Gargioli and Slack, 2004).

Fig.V10



Many cells all over the tadpoles are labelled, but in the muscle the labelling is predominantly in the flat peripheral nuclei of the type expressing Pax7 (Fig.V9A,B). Very few, if any, of the myonuclei are labelled, although a few fibroblast or other cell types within the muscle but outside the myofibres are labelled.

Finally it was investigated whether the regeneration behaviour of the different types of PSM graft correlated with the presence of satellite cells in the grafts. This was done by co-immunostaining sections of grafted tadpoles for Pax7 and for GFP (Fig.V10). Single staining (Fig.V10A,B) showed once again the different pattern of reaction between Pax7 and GFP: Pax7 was expressed in the elongated nuclei and exclusively nuclear, while GFP was expressed mostly in the round nuclei and cytoplasm of the muscle fibres.

Fig.V10 Immunofluorescence with anti-Pax7 (red) and anti-GFP (green) on transverse sections of tadpole myotomes. **(A)** Pax7 in ungrafted tadpole. **(B)** GFP immunoreaction on myotome in PSM grafted embryo. **(C)** Double immunostaining of Pax7 and GFP on muscles from late medial PSM grafted tadpoles. **(D)** Double immunostaining of Pax7 and GFP on muscles from early medial PSM. Co-labelling is seen only in the late grafts. Scale bars 5 μm. (Gargioli and Slack, 2004).

In the double immunostaining it could clearly be seen that the two labels co-localized only in the late medial PSM graft type, which yields labelled muscle in the regenerate (Fig.V10C). No co-localization is shown in tadpole muscle sections for the early medial PSM transplantation, in which muscle is not labelled in the regenerate (Fig.V10D).

Conclusions

In summary the results described in this chapter indicate that the muscles during the tail regeneration process in *Xenopus laevis* follow a characteristic route, similar to the mammalian experimental model. Following the analysis regarding stump muscle fibres, dermis and haemo- mesoangioblast, it is possible to exclude definitely the contribution of those tissue to the regenerating muscle fibres in the re-forming tail. In particular it has been shown that dermal cells contribute to the undifferentiated blastema of the regeneration bud, proliferating but retaining the morphological characteristics of dermal cells. They remain mononuclear, do not fuse and do not form syncytial fibres. The diI experiment indicated that the putative stem cell population associated with the early circulatory system does not participate significantlyin the tail regeneration process, and certainly does not contribute to the re-forming myofibres. The *Cre/lox* system confirmed that the regenerated muscles do not originate by dedifferentiation from the mature pre-existing myofibres of the stump, as haas been shown for urodele amphibians (Newt, Axolotl, ecc.). The evidences from the presomite graft experiments suggest that the regenerated muscles in the reforming tail originate from the GFP/Pax7 positive flat nuclei highlighted in the histological study, and believed to represent muscle satellite cells. They are not formed from the early medial PSM, some arise from the early lateral PSM, and most from the late PSM, which will include tissue from extreme lateral position in the earlier mesoderm because of the process of dorsal convergence during neurulation and somitogenesis. I thus believe that the satellite cells

precursors originate from the lateral region of the PSM. As there is an excellent correlation between the extent of labelling of satellite cells by the three typs of graft, and the extent to which the grafts contribute to myofibres in the regenerate, I believe that the new muscle fibres in the re-forming tail arise from satellite cells. A more detailed study of the origina and lineage of the satellite cells will have to be the object of a new project of work.

VI-Discussion and conclusions

Morphology of the regeneration bud

From the results obtained in this paper we think that the term "blastema" is not appropriate for the whole of the regenerating tissues during *Xenopus* tail regeneration. A "blastema" is usually defined as a mass of undifferentiated pluripotent proliferating cells associated with the epimorphic process of regeneration. In this study we refer to the whole of the regenerating region as a "regeneration bud" and only the visibly undifferentiated tissue within this as a "blastema". The morphological study showed that spinal cord and notochord do not appear to de-differentiate. After amputation they keep their tissue identity, and their own specialised cell populations proliferate to give rise to the regenerating spinal cord and notochord respectively. The spinal cord regenerates from a terminal "ampulla apicalis", formerly described by Stefanelli (1951) in both amphibians and reptiles. This structure is the result of the enlargement of the ependymal canal due to the intense proliferation in the regenerating inner layer of the spinal cord, described in the first chapter by the massive incorporation of BrdU and intense reactivity for PCNA at level of the base of the neural ampulla. In the notochord the cellular population responsible for regeneration appears to be the notochord sheath region. This forms a cellular layer covering the vacuolated cells of the notochord along its length and is continuous with the bullet-shaped mass of cells that forms at the tip during regeneration. The blastema proper is presumably formed by the

fin mesenchyme and dermis as well as from the muscle satellite cells. There is direct evidence for the dermal cell contribution to the blastema formation from the skin graft experiment. This shows the early migration of the dermal cells colonising the blastema and then the intense proliferation of those cells during the tail regeneration process. So they have a role during the tail growth; but do not appear to contribute to the fin mesenchyme. For the regenerated axial tissues only the muscle appears to derive from the blastema, in particular from the muscle satellite cells. On the basis of these findings a schematic view of the regeneration bud is shown in Fig.VI1.

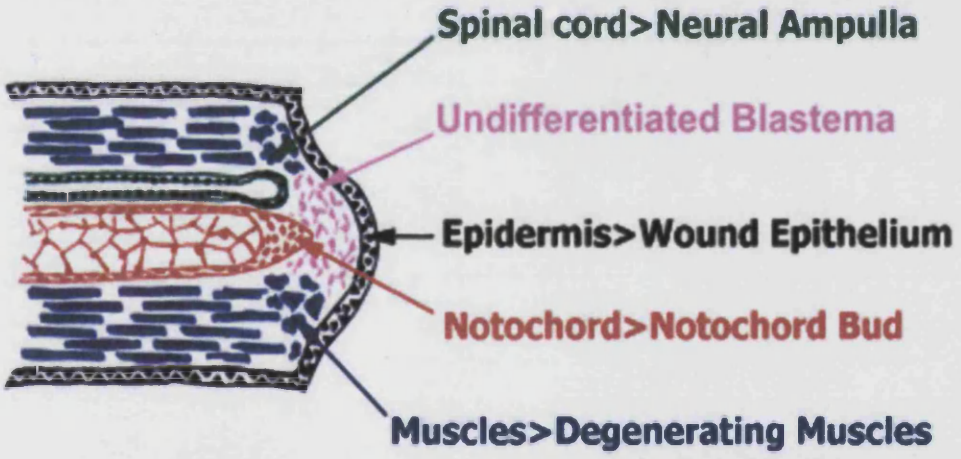


Fig.VI1 Diagram showing the composition of the regeneration bud during *X. laevis* tail regeneration.

Transgenesis and graft

The grafting technique from transgenic neurulae into normal hosts showed specific labelling in the tissue targeted. The transplanted tissue, expressing GFP under control of the CMV promoter, retained fluorescence throughout regeneration and enabled us to follow processes of cellular de-differentiation, migration and re-differentiation. The results for the spinal cord and notochord are straightforward and are consistent with the morphological appearance. Those results show that each of these two tissues regenerates as a self-contained compartment with no export or import of cells, and without de-differentiation or metaplasia. The labelling of the spinal cord was carried out in such a way that only the ventral part was labelled. I did not, therefore, examine the potential of neural crest cells in the tail regeneration process that are derived from the dorsal part of the neural tube. This results is in marked contrast to recent cell labelling study in axolotl tail regeneration in which radial glial cells were shown to give rise to neurons, melanocytes, myofibres and chondrocyte in the regenerate (Echeverri and Tanaka, 2002). I strongly believe that the sets of data obtained are correct in both cases and that the differences reflect the different experimental models used, and the different modes of regeneration in the urodele and anuran amphibians.

Origin of muscle in the regenerate

Before addressing the origin of the myofibres in the regenerate the possibility was considered that the muscle of the regenerate originated not from the myotomes at all but from another mesodermal cellular source such as the

haematopoietic stem cells, or the cells associated with the blood vessels or dermal cells. The entire circulatory system was labelled with LDL-diI (Levine, et al., 2003) but this did not label any myofibres in the regenerate, excluding this hypothesis. By transplanting a segment of skin from transgenic tadpoles expressing CMV-GFP into normal non-transgenic hosts, cells of the epidermis and dermis were labelled and could be followed during the regeneration process. The dermis did contribute to the blastema formation showing cell migration from the stump dermis to the regenerate, nevertheless this did not contribute any cells to the formation of differentiating myofibres in the regenerating muscle of the re-growing tail. These negative results suggest that the regenerated muscle does come from the myotomes, and the evidence presented here suggests that it derives from satellite cells rather than from pre-existing myofibres. Interestingly it has recently been shown that in urodeles as well a considerable contribution to muscle regeneration is provided by satellite cells as well as the de-differentiation of pre-existing mature myofibres of the stump (Tanaka, personal communication).

The evidence for the origin of regenerated muscle from satellite cells ratehr than myofibres is as follows. First, the original myofibres can seen to be degenerating in the vicinity of the cut surface. Second, myofibres labelled by the Cre-Lox technique do not contribute to myofibres in the regenerate in the short term. The small numbers of labelled fibres that do appear arise late and this is consistent with new differentiation of muscle fibres whose time course can be seen in the *Car-GFP* transgenic. Ryffel et al. (2003) also used the Cre-Lox method to label myofibres, using a protocol involving breeding, and also

showed that labelled muscle cells did not enter the myofibres of the regenerate. Third, myofibres labelled by the early medial PSM grafts never contribute muscle fibres in the regenerate. Fourth, the tadpole myotomes contain cells, identified by Pax7 expression, with the morphology of muscle satellite cells. Fifth, these cells are the proliferating cells within the myotomes of non-regenerating tadpoles as shown by the BrdU incorporation. Sixth, for the three types of PSM graft, the labelling of myofibres in the regenerate is correlated with the number of the satellite cells labelled by the graft. The different behaviour of the PSM grafts is fully consistent with the idea that the satellite cells precursors lie in the far lateral part of the PSM. During somitogenesis there is a strong dorsal convergence of cells from the ventral side of the embryo (Pourquiè, 2001). The PSM in the early neurula is not much thicker than the ventral mesoderm, but by the late neurula it has become markedly thicker, at the expense of the ventral mesoderm, and segmentation has commenced at the anterior end (Nieuwkoop and Faber, 1967). I believe that the early lateral grafts capture some of the satellite cell precursors but that the late medial grafts capture more because of the substantial migration of tissue towards the dorsal midline that occurs in this stage interval. In newt limb regeneration it has clearly been shown that the nuclei of myofibres can re-enter S phase following a nearby injury, that the fibres can break up into viable mononuclear cells, and that that these cells can re-differentiate to form new muscle fibres and also make some contribution to other cells tupes (Kintner and Brockes, 1984; Echeverri et al., 2001; Echeverri and Tanaka, 2002). My results on the origin of the

regenerated muscle is quite different from these published results from urodeles. However recently an oral report indicated that in urodeles there is also a contribution from the satellite cells to muscle regeneration (Tanaka, personal communication).

Muscle satellite cells were first decribed by Mauro (1961), who provided EM pictures of cells from the frog very similar in appearance to the flat nucleus cells found labelled by the late PSM graft. Subsequent work has all been on birds and mammals (reviewed by Seale and Rudniki, 2000), although it is likely that amphibian satellite cells have similar properties. Following muscle damage, satellite cells re-enter mitotis and start to express myogenic transcription factors, and the resulting myoblasts fuse with each other to generate new myofibres (Shultz, 1996; Cooper et al., 1999). The formation of mammalian satellite cells, but not primary or secondary myofibres, is now known to depend on the transcription factor Pax7 (Seale et al., 2000). They have been previously shown to originate from the somites in birds, using quail-chick grafting (Armand et al., 1983), although more recent work suggests they may arise from the aorta (De Angelis, et al., 1999). As the dorsal aorta is now known to be colonised by a population of somatic angioblasts (Pardanaud et al., 2000), these findings are not necessarily inconsistent. My results indicate that *Xenopus* has satellite cells similar to those in birds and mammals, that they express Pax7, and that they arise from lateral region of the PSM.

Conclusions

The regeneration of the *Xenopus* tadpole tail operates through mechanism that are completely different from those found in the appendage regeneration of urodeles. In urodeles functional cells de-differentiate to form a blastema. This proliferates and re-differentiates to form the regenerate. There is a certain amount of metaplasia shown indicating that at least some of the blastema cells show pluripotency. In *Xenopus* the spinal cord regenerates through the proliferation of an apical ampulla continuous with the ependymal layer of the more proximal spinal cord. The notochord regenerates by expansion of the sheath cell population in to a proliferating tip. The growth of the notochord has not been much studied in mammals but it is likely that the sheath cells are the precursor of the highly differentiated vacuolated cells in the centre of the notochord. The muscle regenerates by the multiplication, differentiation, and fusion of the satellite cells to form new myofibres. I can speculate regarding this process and say that probably the growth of the myofibres, in post- embryonic life , could carry on with the proliferarion and fusion of the muscle satellite cells, as in the muscle growth of mammals. Overall, *Xenopus* tail regeneration seems much more akin to the normal tissue renewal mechanism found in mammals than to the specialised regeneration mechanism found in the uordeles (Carlson, 2003). This may make *Xenopus* a more useful model organism than formerly suspected for experimental work in regenerative medicine.

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